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are thought to be mediated by the localized release of *S aureus* exfoliative exotoxin.[3]

In this case, a 32-year-old white man with redness, purulent discharge, and grittiness in the left eye for 3 days was examined. He reported the onset of erythematous and pustular lesions over the entire face, particularly on the left. This was the fifth such episode that involved the eye and skin in the last 3 years; however, on this occasion, the facial rash was more prominent, and the right eye was not affected. Clinical examination revealed visual acuity of 20/20 in the right eye and 20/30 in the left eye. There were erythema and edema of the left eyelids, marked papillary conjunctivitis (Figure 1), purulent discharge, and palpable preauricular lymph nodes. The remainder of the ocular examination was unremarkable. There were multiple erythematous lesions, superficial thin walled bullae, and superficial erosions covered by yellowish-brown colored crusts (Figure 2). These were on the left face predominantly and crossed the mid line.

Swabs were taken from the left conjunctiva, skin lesions, and the nose; and the patient was placed on topical chloramphenicol ointment to the left eye and oral dicloxacillin (2 g/d). Methicillin-sensitive *S aureus* was grown in all cultures, and polymorphs were seen on microscopy. The patient had an elevated white cell count at 13.9×10^9/L with a predominant neutrophilia. Treatment with chloramphenicol ointment and dicloxacillin was continued for 10 days. During this time, visual acuity returned to 20/20 in his left eye, with complete resolution of the conjunctivitis and impetigo. There was no recurrence over a 4-month follow-up period.

Bacterial swabs that are taken from the infected sites before treatment are important in the identification of the exact pathogen and ensure treatment with an antibiotic to which the organism is sensitive.[1] In addition, it is imperative for the diagnosis of nasal carriage of staphylococcus in the setting of recurrent symptoms, nasal carriage of the organism may allow recurrent inoculation of the conjunctiva and skin.[2] Intranasal antibiotic ointment (such as mupirocin) can significantly reduce the rate of nasal carriage of *S aureus* in recurrent and resistant cases.[5] The treatment of recurrent infection may also include a course of oral antibiotics (for example, clindamycin or rifampicin),[5] antiseptic body wash, and the daily washing and disinfecting of bed linen, towels, and clothing. There is little evidence, however, regarding the efficacy of these latter strategies.

This case demonstrates an uncommon association between bullous impetigo and recurrent conjunctivitis. It highlights the importance of examining the face for skin lesions and the need to take bacterial swabs from the nose. The diagnosis of nasal carriage of *S aureus* has important treatment implications. Systemic antibiotics and topical mupirocin can decolonize the nose and reduce the recurrence of infection.

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The Persistence of Transplanted Amniotic Membrane in Corneal Stroma

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PURPOSE: To characterize the long-term incorporation of transplanted amniotic membrane into corneal stroma.
DESIGN: Experimental study.
METHODS: Rabbit amniotic membrane, stained with a fluorescent dye (DTAF), was implanted unilaterally into the corneal stroma of four adult rabbits. Corneas were examined clinically and by transmission electron microscopy and fluorescent microscopy at 1, 3, 7, and 10 months after surgery.
RESULTS: Visibility of the transplanted amniotic membrane, in situ, on slit-lamp examination decreased over time. However, fluorescent and electron microscopy clearly demonstrated that the amniotic membrane remained structurally unchanged and intact within the corneal stroma up to 10 months after implantation.
CONCLUSIONS: Amniotic membrane allografts persist intact within an intracorneal space for many months postoperatively and are not quickly broken down or dissolved by the host tissue. (Am J Ophthalmol 2006; 141:190–192. © 2006 by Elsevier Inc. All rights reserved.)

HISTORICALLY, THE AMNIOTIC MEMBRANE HAS BEEN used as a biologic membrane to treat burns and ulcers of the skin,[1] and more recently, it has proven to be a

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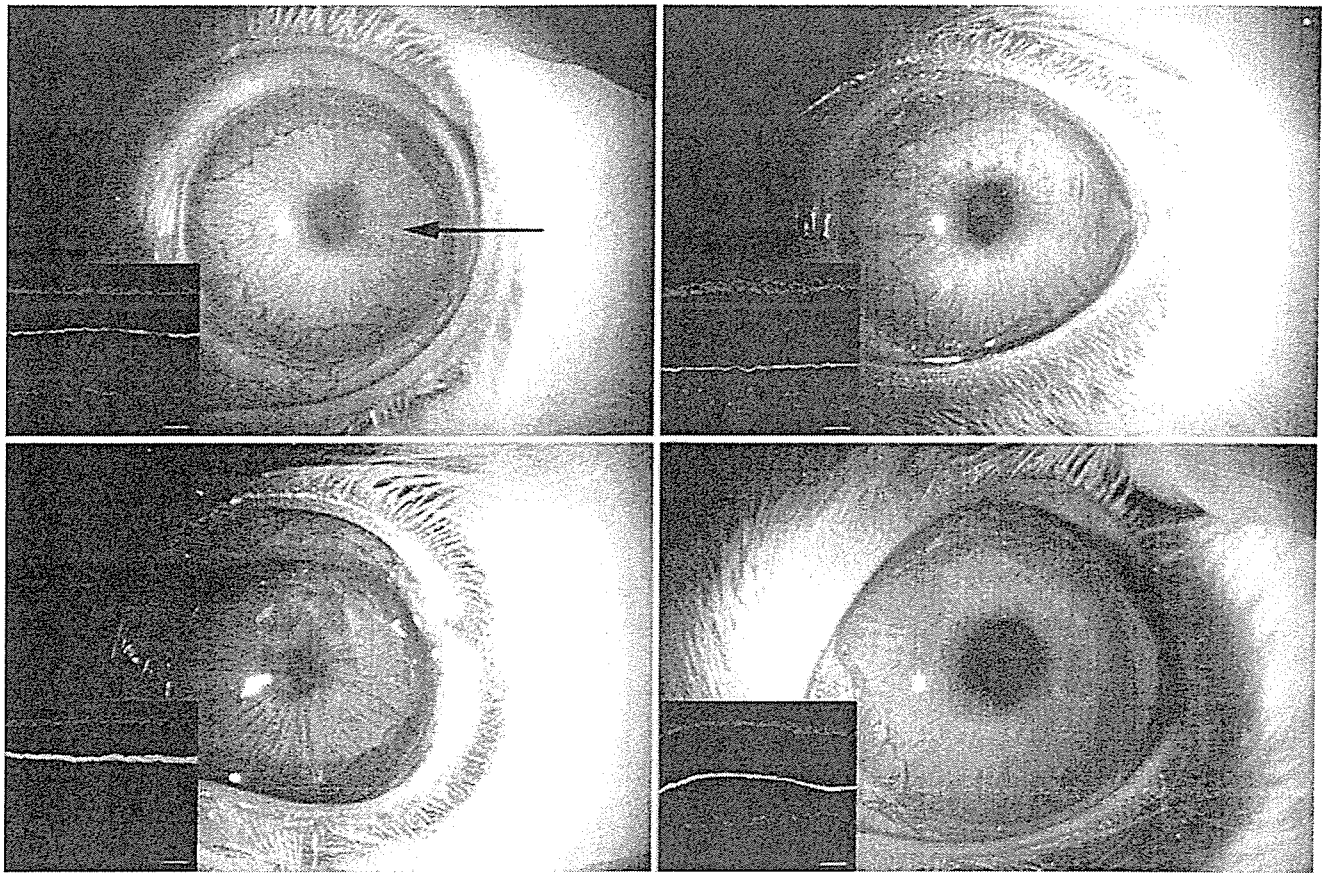

FIGURE 1. Transplanted amniotic membrane in corneal stroma with photographs of the right rabbit eyes before enucleation. (Top left) the transplanted amniotic membrane was still visible at 1 month (arrow). Visibility improved progressively at 3 (top right), 7 (bottom left), and 10 months (bottom right). However, fluorescence microscopy (inserts) clearly demonstrated that the DTAF stained amniotic membrane (amniotic membrane = green, corneal nuclei = red) persisted throughout the study. Scale bars = 200 μm).

valuable tool in the management of ocular surface disorders.[2,3] The successful use of amniotic membrane in ocular surgery is thought to be attributable to its anti-inflammatory, antiangiogenic, and antibacterial properties, as well as its resultant transparency.[3–5] However, despite favorable clinical results, the relationship between the host tissue and the transplanted amniotic membrane after surgery remains controversial, and any influence the corneal stroma may have on amniotic membrane structure has yet to be properly characterized. We, therefore, examined the long-term fate of amniotic membrane after its transplantation into the corneal stroma of a quiescent eye using an allotransplantation rabbit model.

Four adult New Zealand white rabbits weighing 2.5 to 3.0 kg with clinically normal eyes were used in the experiment and treated in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. Rabbit amniotic membrane was separated from the chorion, thoroughly washed in phosphate buffered saline (pH 7.2), and cut into 5 mm × 5 mm pieces that were incubated in 0.5% dichlorotriazinyl aminofluorescein (DTAF) in 0.2 mol/l sodium bicarbonate for 1 minute. DTAF stained membrane was thoroughly rinsed in phosphate buffered saline before use to ensure any unbound stain was removed. DTAF is a fluorescent dye, which binds covalently to collagen under physiologic conditions enabling its location in living tissue to be easily and accurately traced for up to a year following staining.[6]

Within each of the rabbits' eyes a mid-depth central stromal pocket, parallel to the corneal surface and measuring 6 mm in diameter with a 2-mm circumferential opening at its edge was fashioned. Into this one piece of DTAF-treated amniotic membrane was carefully inserted. The wound was left to heal unsutured, and antibiotics (Ofloxacin, 400 mg) were added drop-wise twice a day for 5 days. After 1 month, the cornea remained hazy in and around the position of the implanted amniotic membrane, however, visibility gradually increased over time finally resulting in a clear cornea by 10 months (Figure 1).

Eyes were enucleated at 1, 3, 7, and 10 months following surgery and processed for fluorescence and electron micros-

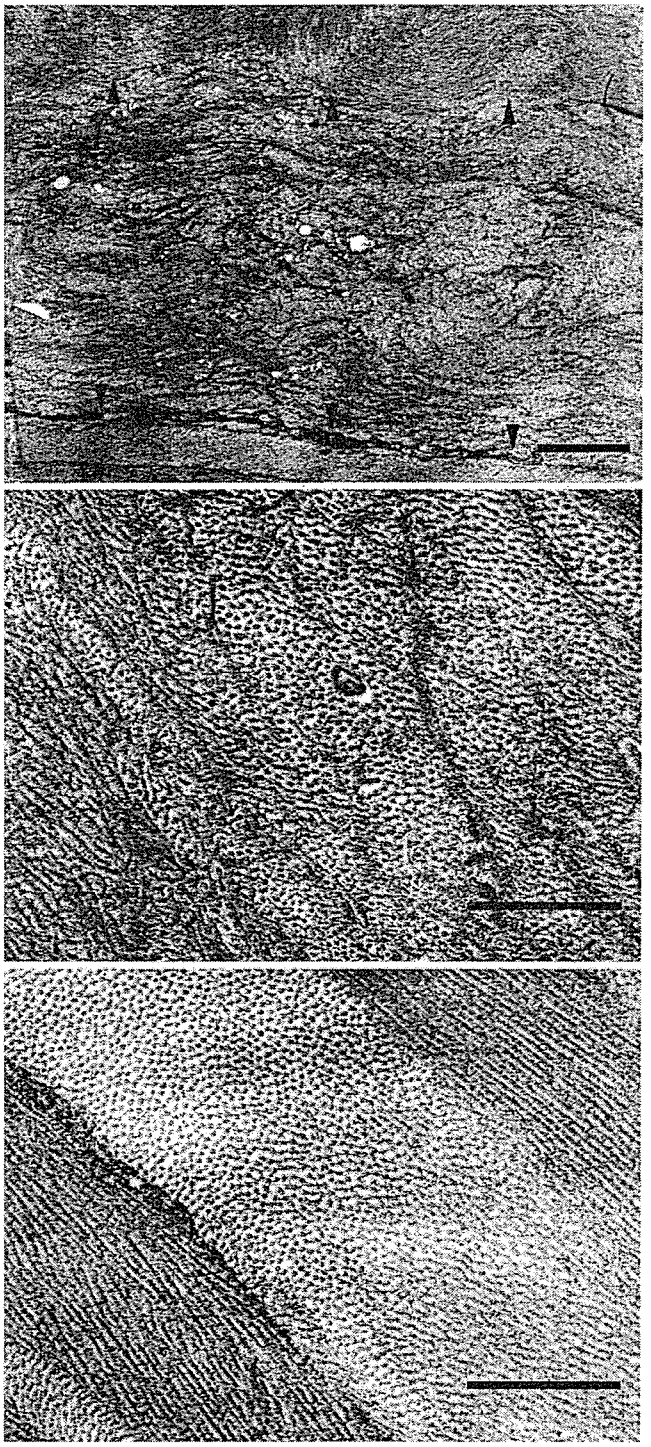

FIGURE 2. Transmission electron microscopy of the amniotic membrane and corneal stroma. (Top) No obvious change to the implanted amniotic membrane ultrastructure was observed at 10 months (arrowheads denote inside edge of amniotic membrane). Scale bar = 3 μm. (Middle) High magnification image of disorganized collagen fibrils within implanted amniotic membrane 10 months postoperatively. Scale bar = 1 μm. (Bottom) High magnification image of regularly aligned collagen fibrils from an area of the corneal stroma adjacent to the implant. Scale bar = 1 μm.

copy. For fluorescence microscopy half of each cornea was embedded in OCT, snap frozen in liquid nitrogen, sectioned (6 μm), and counterstained with propidium iodide. The remaining halves were fixed in glutaraldehyde 2.5%, dehydrated, embedded in Araldite, sectioned, and counterstained with lead citrate and uranyl acetate before examination in a transmission electron microscope. Using fluorescence microscopy, the DTAF stained amniotic membrane was consistently located within sections through the stroma at all time points examined and retained a similar level of fluorescence, thickness, and location throughout (Figure 1). The constant intensity and concentration of the fluorescent stain suggested that considerable bleeding of DTAF into the surrounding stroma did not occur, because this would have resulted in a more diffuse and less intense staining pattern over time. Transmission electron microscopy revealed the ultrastructure of both the host stroma and the transplanted amniotic membrane to be essentially unchanged (Figure 2).

This study indicates that amniotic membrane, once transplanted into the corneal stroma, can remain intact within the cornea for many months postoperatively without being broken down or dissolved by the host tissue. However, its continued presence within the eye does not result in inflammation, rejection, or a loss of transparency. Therefore, amniotic membrane is highly suitable for the surgical reconstruction of the corneal stroma.

ACKNOWLEDGMENTS

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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.)

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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

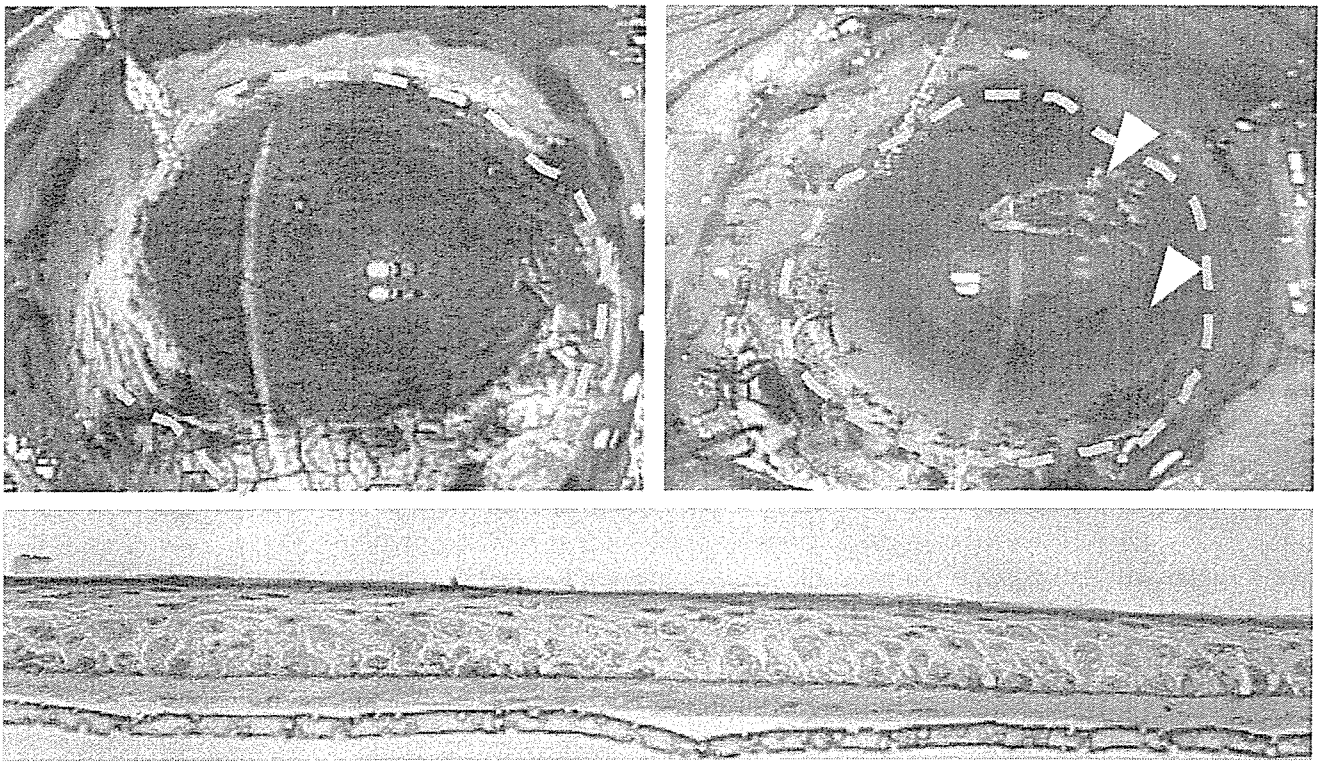

FIGURE 1. The integrity of all transplanted cultivated oral mucosal epithelial sheets confirmed by fluorescein staining at the end of ocular surface reconstruction. The yellow broken line encompasses negatively-stained cultivated stratified oral mucosal epithelium. Arrows indicate the region harboring 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

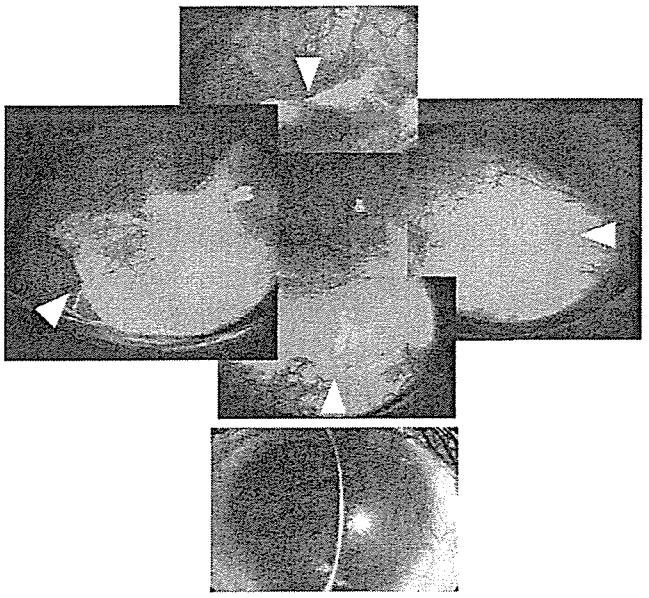

FIGURE 2. The clinical appearance of case 1 at 34 months after cultivated autologous oral mucosal epithelial transplantation. Fluorescein staining confirms the long-term survival of oral mucosal epithelium identified by the different levels of staining density. Arrows indicate the margin of the outgrowth 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.

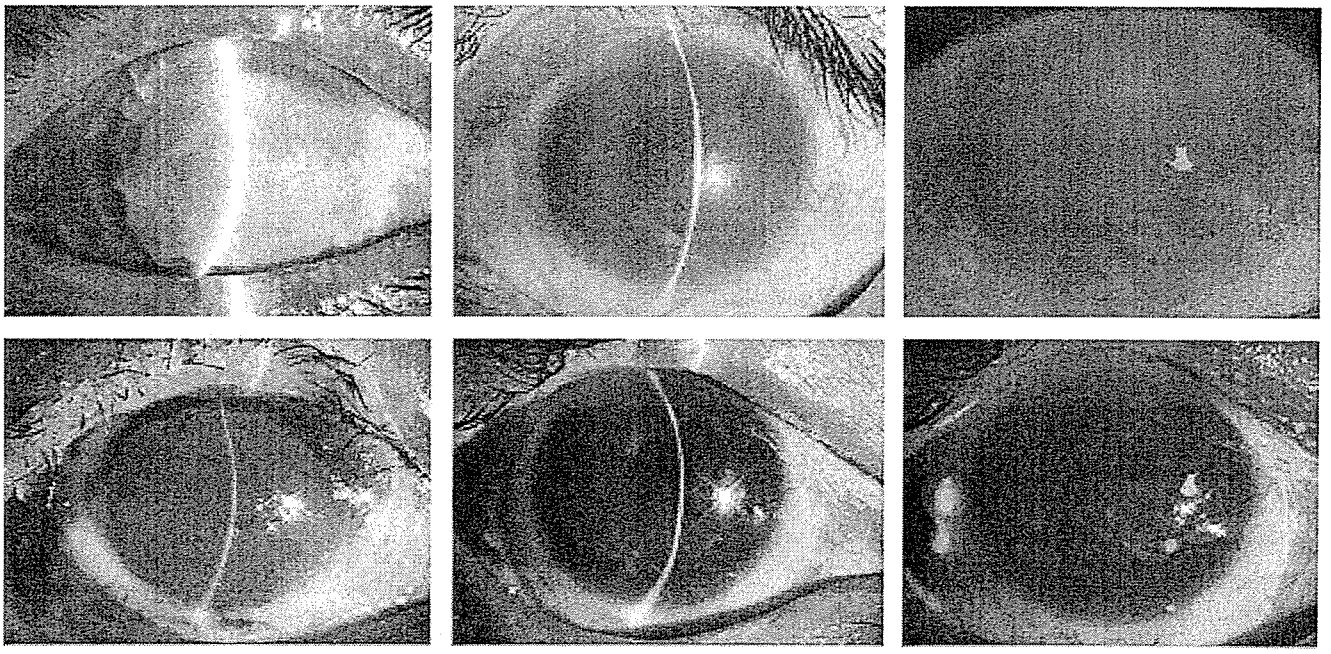

FIGURE 3. Slit-lamp photographs of two patients in the acute phase of chemical/thermal injury shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 1 (33-year-old man): acute phase of alkali injury graded IIIb with severe corneal stromal opacity. (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

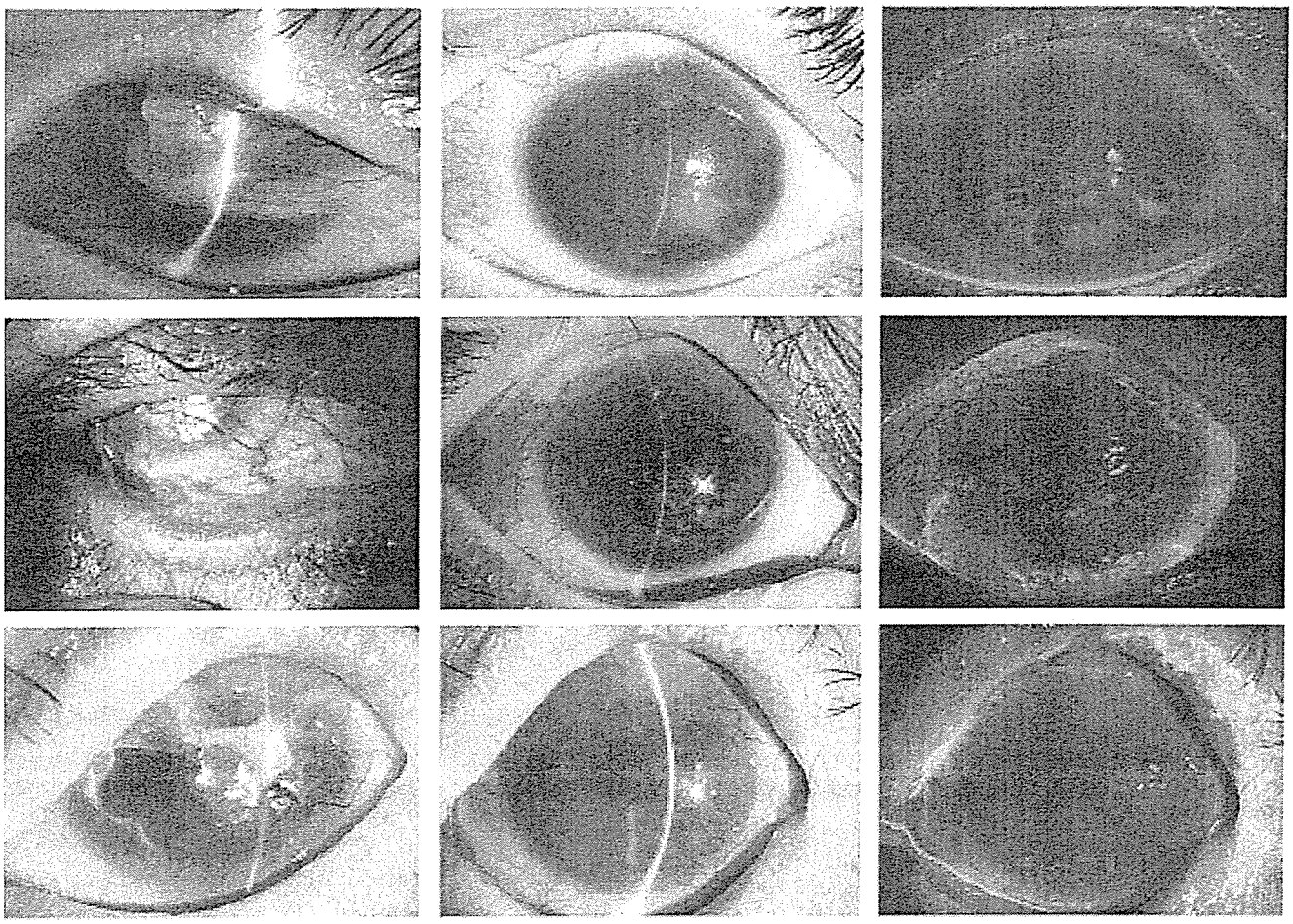

FIGURE 4. Slit-lamp photographs of three patients in the chronic phase of ocular surface disorders shown before and after ocular surface reconstruction using cultivated oral mucosal epithelial transplantation. Case 5 (14-year-old girl): chronic phase of SJS with severe symblepharon over the cornea. (Top 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-

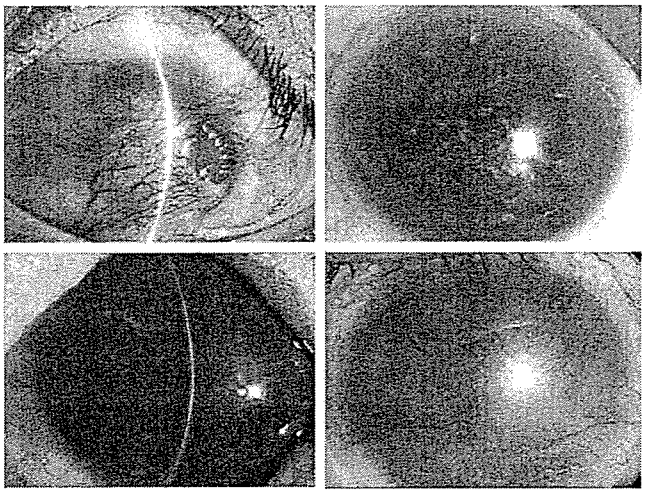

FIGURE 5. Slit-lamp photographs showing vascular formations after the transplantation of cultivated autologous oral mucosal epithelial sheets. (Top left) Preoperatively, most of the cornea manifested highly-vascularized conjunctivalization (case 3, grade 4). At the 1-year follow-up, vascular formations 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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REPORTING VISUAL ACUITIES

The AJO encourages authors to report the visual acuity in the manuscript using the same nomenclature that was used in gathering the data provided they were recorded in one of the methods listed here. This table of equivalent visual acuities is provided to the readers as an aid to interpret visual acuity findings in familiar units.

Table of Equivalent Visual Acuity Measurements

Snellen Visual Acuities				
4 Meters	6 Meters	20 feet	Decimal Fraction	LogMar
4/40	6/60	20/200	0.10	+1.0
4/32	6/48	20/160	0.125	+0.9
4/25	6/38	20/125	0.16	+0.8
4/20	6/30	20/100	0.20	+0.7
4/16	6/24	20/80	0.25	+0.6
4/12.6	6/20	20/63	0.32	+0.5
4/10	6/15	20/50	0.40	+0.4
4/8	6/12	20/40	0.50	+0.3
4/6.3	6/10	20/32	0.63	+0.2
4/5	6/7.5	20/25	0.80	+0.1
4/4	6/6	20/20	1.00	0.0
4/3.2	6/5	20/16	1.25	−0.1
4/2.5	6/3.75	20/12.5	1.60	−0.3
4/2	6/3	20/10	2.00	−0.3

From Ferris FL III, Kassoff A, Bresnick GH, Bailey I. New visual acuity charts for clinical research. Am J Ophthalmol 1982;94:91–96.

Unique Distribution of Thrombospondin-1 in Human Ocular Surface Epithelium

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Purpose. The study was conducted to elucidate the detailed expression pattern of angiogenesis-related factors in human ocular surface epithelium. The focus was factors with significantly higher gene expression in corneal epithelium (CE) than in conjunctival epithelium (CJE).

Methods. The relative gene expression of 36 angiogenesis-related factors was compared in human CE and CJE, by using the introduced amplified fragment-length polymorphism (iAFLP) method. Also examined were the expression patterns in the CE, limbal epithelium (LE), and CJE of factors with significantly higher expression in the CE, by using real-time PCR, in situ hybridization, immunohistochemistry, and immunoelectron microscopy.

Results. Only thrombospondin (TSP)-1 exhibited significantly higher expression in the CE. In situ hybridization and real-time PCR showed TSP-1 transcripts in the basal cells of the CE and LE. Compared with the CJE, they were significantly upregulated at those sites. Immunohistochemistry revealed that TSP-1 was strongly expressed in the basal region of the CE. Its expression was faint in LE and absent in CJE. Immunoelectron microscopy revealed that the CE and LE demonstrated TSP-1 labeling just below the epithelium, in the basal region of basal cells, and occasionally in the basal cell membrane. There was little or no labeling in the CJE.

Conclusions. In the human ocular surface epithelium, basal cells of the CE and LE, but not of the CJE, synthesize TSP-1. High levels of TSP-1 are present only just below the CE. Its unique distribution may be related to corneal avascularity and integrity. (*Invest Ophthalmol Vis Sci.* 2006;47:1352–1358) DOI:10.1167/iovs.05-1305

The corneal epithelium (CE) and conjunctival epithelium (CJE) are continuous, and together they comprise the ocular surface. Although both are stratified, squamous, nonkeratinizing epithelia that originate from surface ectoderm, they differ considerably in their characteristics and functions. For example, although some keratins are expressed in the CE and CJE as cytoskeletal proteins, they are of different subtypes.[1,2] In the CJE, but not the CE, goblet cells are interspersed between epithelial cells, and the tight junctions of the superficial cells render the CE impermeable to water-soluble substances. Consequently, compared with the CE, the CJE exhibits very poor barrier function.[3,4]

One of the major differences between the CE and CJE is the underlying vascular system. Although the connective tissue under the CJE is rich in vessels, the tissue under the normal CE is devoid of a vascular system. Corneal avascularity, necessary for good visual acuity, is attributable to the balanced expression of angiogenic and antiangiogenic factors.[5–10] Although the cornea is thought to produce and contain numerous antiangiogenic factors including thrombospondins,[5] pigment epithelial-derived factor,[6] and endostatin,[7] the mechanisms underlying the maintenance of corneal avascularity remain poorly understood.

To gain insight into angiogenesis-related corneal characteristics, we compared the gene expression patterns of 36 factors related to angiogenesis in the CE and CJE. We then investigated the detailed expression patterns in the ocular surface epithelium of factors with significantly higher expression in the CE than the CJE.

Materials and Methods

RNA Extraction from CE, LE, and CJE

Our study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. Human conjunctival epithelial cells were obtained from donors who provided prior informed consent.

Using donor tissues obtained from the Northwest Lions Eye Bank (Seattle, WA), we scraped the 4-mm diameter central region of the epithelium to obtain CE. For limbal epithelium (LE) we cut out the center part of the cornea and scraped the epithelium corresponding to the region of the palisades of Vogt (n = 11; mean age, 57.5 ± 6.4 years). After scraping the LE, we prepared sections of the remaining tissues and stained them with hematoxylin and eosin to ascertain that we harvested only LE and not CE. CJE cells from the bulbar conjunctiva of healthy volunteers were collected under topical anesthesia with 0.4% oxybuprocaine eye drops using a sterile nylon thread brush (Cytobrush Plus; Medscard Medical AB, Malmo, Sweden), as previously described (n = 6; mean age, 52 ± 5.8 years).[11]

After a 5-minute immersion of the CE, LE, and CJE at room temperature (RT) in acid phenol with guanidine isothiocyanate (TRIZOL reagent; Invitrogen Corp., Carlsbad, CA), total RNA was extracted and dissolved in distilled water according to the manufacturer's guidelines. To check its integrity, 1 μL of the RNA solution was electrophoresed on 1% agarose gels, the gels were stained (SYBR Green; Molecular Probes Inc., Eugene, OR), and 18S and 28S ribosomal RNA band fluorescence was quantified with a luminescent image analyzer (LAS1000; Fuji Film Medical Systems Inc., Stamford, CT). The remaining RNA was stored at −80°C until use.

Gene Expression Analysis by iAFLP

We examined the gene expression patterns in the CE and CJE of 36 angiogenesis-related factors (Table 1), using the introduced amplified fragment length polymorphism (iAFLP) method,[12,13] described else-

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TABLE 1. Angiogenesis-Related Factors and Gene-Specific Primers

Thrombospondin-1*	5′-GTGATGAGTAAGGGTGGGGA-3′	TNF-alpha1*	5′-GCCAGATTCCAGATGTCAGGG-3′
Thrombospondin-3*	5′-GCCTCGCATGGATGTGTCAAT-3′	TNF-alpha2*	5′-GCCAAGGAGGAGTCATATCT-3′
VEGF	5′-GTCTTTCTGTCCGTCTGACCGT-3′	HGF	5′-GTAAGGCTTTTTAGTATAAGA-3′
FGF-1	5′-GCAAAGCAGGTCCGCAGATGA-3′	METH-1*	5′-GCCCCACCACAAGACAAGTGA-3′
FGF-2	5′-GTCCTACCTATTCCTTAAACT-3′	METH-2*	5′-GATGAAGTGAGGTCTGTTGA-3′
Angiopoietin-1	5′-GAGTAATGGAGTTTGTTTCTCA-3′	SDF-1	5′-GATACCACCAGGACCTTCTGT-3′
Angiopoietin-2	5′-GAGAATGCAGTTCGAAGATGA-3′	NK 4*	5′-GTGTCCCCAGGCTCCTCGGTT-3′
IL-1 alpha*	5′-GATGTAACATTATGGTCTGA-3′	GRO-beta*	5′-GCACACATACATTTCCCTGCC-3′
IL-1 beta*	5′-GCCTGGCTGATGGACAGGAGA-3′	IP-10*	5′-GCCCTCTGGTTTTAAGGAGAT-3′
IL-8*	5′-GTGGAACAAGGACTTGTGGA-3′	B 61	5′-GTAGCTCCTGCCGTGTGGTGA-3′
IL-12 alpha	5′-GTCCACAGTAAATGTCAAAAATACT-3′	PF 4	5′-GCTATCAGTTGGGCAGTGGG-3′
IL-12 beta	5′-GTTTGCATAATAGGGACTGAT-3′	Somatostatin	5′-GGAAGAGAGATGGGGTGTGG-3′
TGF-beta 1	5′-GCAGCGTTGACGTCCCAGGAT-3′	Haptoglobin	5′-GAGCTCTTATCAAAGCTTAAGA-3′
TGF-beta 2*	5′-GCGTGTATCCATTTCCACCCT-3′	Erythropoietin*	5′-GTGGGGCCATTAGTTCCAGA-3′
TGF-beta 3*	5′-GTGTCCAAGGGGAAATATGA-3′	TIMP-1*	5′-GATACATCTTGGTCATCTTGA-3′
IGF	5′-GCATAGTTCATTAACTCTTTTACCA-3′	TIMP-2	5′-GTCAGGCCCTGCCCTAACCCA-3′
Angiogenin*	5′-GACGACGGAAAATTGACTGA-3′	G-CSF	5′-GTCCAAGGTGGGACCCAACTC-3′
Angiogenin-inhibitor*	5′-GACTGGCAGAAATAATCGGA-3′	CHM-1	5′-GCCCCCCACGGGTTCACAGA-3′

*Factors yielding products of the expected sizes.

where (n = 6). For validation and normalization, we examined the gene expression patterns of four keratins, 19 ribosomal protein subunits, and GAPDH by the same method. We used a pUC 19-base vector primer[14] to monitor the adequate synthesis and cleavage of cDNA on agarose gels. Extracted total RNA (1 μg) was annealed with a pUC 19-base vector primer (5 ng) in a total volume of 10 μL. After heat denaturation (70°C, 3 minutes), the reaction mixture that included 0.5 mM dNTP, 1× RT buffer (supplied with SuperScript II; Invitrogen), 15 mM dithiothreitol (DTT), and 0.1 U/mL of reverse transcriptase (SuperScript II; Invitrogen) was incubated for 60 minutes at 42°C. Second-strand synthesis then followed, in which 130 mL of second-strand reaction mixture was added, to yield a final concentration of 0.33 mM dNTP, 2.7 mM DTT, 1× *Escherichia coli* ligase buffer (supplied with *E. coli* ligase), 0.27 U/mL DNA polymerase I, 0.13 U/mL *E. coli* ligase, and 0.013 U/mL *E. coli* RNase H (all from Invitrogen). After purification by phenol-chloroform extraction and ethanol precipitation, cDNA was dissolved in 20 mL of distilled water, and 2.5 mL of the solution was stocked for electrophoresis.

To digest cDNA, 2 mL of 10× NEB3 buffer (supplied with *MboI*) and 5 units of *MboI* (New England Biolabs Ltd., Herfordshire, UK) were added. This was followed by incubation at 37°C for 60 minutes and heat inactivation at 70°C for 20 minutes. A small aliquot of the digested cDNA was electrophoresed on agarose gels along with stocked undigested cDNA, to check the quality of cDNA synthesis and *MboI* digestion. After checking, small aliquots (approximately 1:6) of all digested cDNAs were pooled to obtain a reference sample (control) to connect the data among the different sample sets. Then 100 picomoles of proper length-polymorphic (LP) adaptor (LP40, control; LP43, sample 1; LP46, sample 2; LP49, sample3; LP52, sample 4; LP55, sample 5), adenosine triphosphate (ATP), T4 DNA ligase (New England Biolabs, Ltd.), and 10× T4 ligase buffer (supplied with T4 DNA ligase, final concentration 1×) was added to the reaction mixture. After a 3-hour incubation at 16°C, all samples were pooled. They served as the template for PCR profiling. Using each template in the same ratio, PCR was performed with a gene-specific primer and a dye-labeled adaptor primer.[12] The conditions were preincubation at 94°C for 3 minutes and final extension at 72°C for 10 minutes, 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR product was mixed with formamide loading buffer containing 1:4 volume of TAMRA-labeled size marker (GeneScan 350 TAMRA; Applied Biosystems [ABI], Foster City, CA). The mixture was denatured at 94°C for 2 minutes and then electrophoresed on a 10% acrylamide gel containing 6 M urea at a well-to-detector distance of 15 cm. We used an autosequencer (Prism377XL; ABI) and fragment analysis software (Genescan analysis software; ABI) to quantify each dye-labeled amplified fragment. Raw data were normalized by dividing the peak height of each target gene by the corresponding GAPDH gene peak height.

Real-Time PCR

We performed real-time PCR for TSP-1 (Prism 7000; ABI; n = 5). For reverse transcription we used 1 μg of total RNA, oligo (dT) primer, and RNase H-reverse transcriptase (SuperScript II; Invitrogen). Primers and probes for TSP-1 and β-actin were purchased from ABI. For relative quantification we used the ΔC_T method (ABI).[15] The C_T value is the fractional cycle number at which the amplified target amount reaches a fixed threshold of detectable fluorescence. The threshold was set in the midlinear phase of the amplification plot. To standardize the amount of sample cDNA added to each reaction, the amount of target gene in each sample was normalized to the endogenous control (β-actin) by subtracting the C_T of β-actin from the C_T of the target gene. Analyses were performed in a sequence detector (Prism 7000; ABI) using the accompanying data-analysis software.

In Situ Hybridization

Human TSP-1 cDNA, corresponding to base pairs 3874-4375 of the previously reported human TSP-1 cDNA (GenBank accession number NM_003246; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), was subcloned into the pGEM-T vector (Promega, Madison, WI) and used for the generation of sense and antisense RNA probes. Digoxigenin (DIG)-labeled RNA probes were prepared with DIG RNA labeling mix (Roche, Basel, Switzerland).

Fresh human corneal-, limbal-, and conjunctival epithelia obtained from the eye bank were dissected, fixed, and embedded in paraffin by using proprietary procedures, and 6-μm sections were cut (n = 3; eyes aged 36.7 ± 16.5 years). They were deparaffinized with xylene, rehydrated with an ethanol series and phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (15 minutes), and then washed with PBS. Then the sections were treated with 10 μg/mL proteinase K in PBS (30 minutes, 37°C), washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 M HCl for 10 minutes. After they were washed with PBS, the sections were acetylated by a 10-minute incubation in 0.1 M triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride. After they were again washed with PBS, they were dehydrated through an ethanol series. After 55°C, 16-hour hybridization to probes (100 ng/mL) in probe diluent (Genostaff, Tokyo, Japan), the sections were washed in 5× hybridization buffer equal to 5× SSC (HybriWash; Genostaff) at 55°C for 20 minutes and then in 50% formamide, 2× hybridization buffer (55°C, 20 minutes; HybriWash, Genostaff). This was followed by RNase treatment in 50 μg/mL RNase A in 10 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM EDTA. The sections were washed twice with 2× hybridization buffer (55°C, 20 minutes), twice with 0.2× of the buffer (55°C, 20 minutes), and once with TBST (Tris-buffered saline-0.1% Tween-20). After 30-

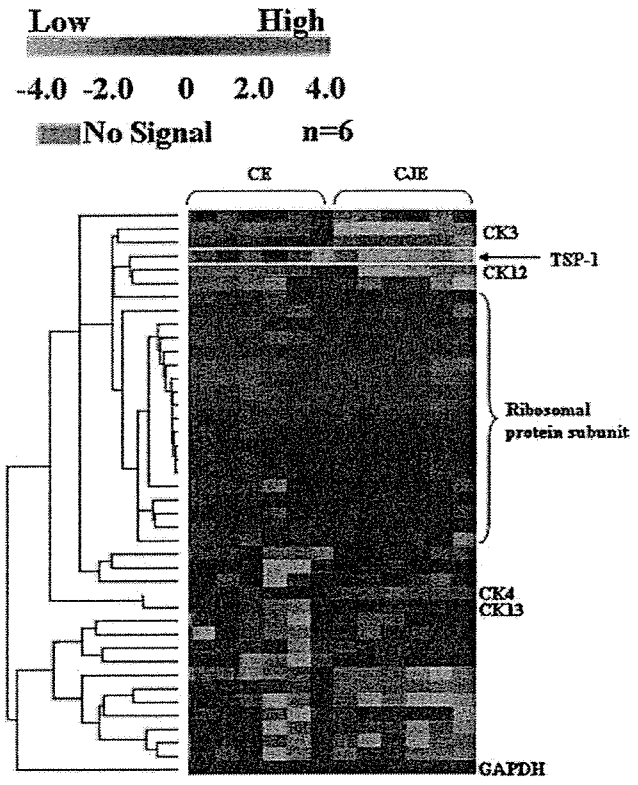

FIGURE 1. Hierarchical clustering of gene expression data obtained with the iAFLP method (n = 6). Each row represents an individual gene and each column an individual sample. Data are presented according to the color scale shown at *top left*. CK3, -4, -12, and -13 were used for validation in this experiment. TSP-1 (*yellow box*) was upregulated in the CE and was clustered close to CK3 and -12, which were also upregulated in the CE. CK4 and -13 were upregulated in the CJE and closely clustered. The ribosomal protein subunits showed similar expression patterns.

minute treatment with 0.5% blocking reagent (Roche) in TBST, the sections were incubated for 2 hours with anti-DIG AP conjugate (Roche) diluted 1:1000 with TBST. They were then washed twice with TBST and incubated in 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, and 100 mM Tris-HCl (pH 9.5). After an overnight color reaction with the BM purple alkaline phosphatase (AP) substrate (Roche), the sections were washed with PBS, dehydrated, and mounted on coverslipped glass slides (Malinol; Mutoh Chemical Co., Tokyo, Japan).

Immunohistochemistry for TSP-1

Residual tissues from penetrating keratoplasty (PKP) were obtained from the eye bank (n = 5, mean age: 49 ± 17.3 years). They were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles, Inc., Elkhart, IN) and 8-μm-thick cryostat sections were cut so that the sections contained continuous epithelium from the central cornea to the limbal conjunctiva. The samples were examined immunohistochemically using our previously described method.[16] Briefly, after a 10-minute fixation in cold acetone, the sections were incubated for 20 minutes with 10% goat serum and 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), incubated again at RT for 1 hour with two types of mouse anti-human primary antibody (catalog no. BA18, dilution: ×100; Oncogene, San Diego, CA; catalog no. T2905; dilution, ×100; Sigma-Aldrich), and washed three times for 10 minutes each in PBS. In control experiments, we replaced the primary antibody with identical concentrations of appropriate nonspecific normal mouse IgG (Dako, Kyoto, Japan).

After incubation with the primary antibody, the sections were washed with PBS containing 0.15% Triton X-100 and then incubated for 1 hour at RT with the appropriate secondary antibodies, FITC-conjugated anti-mouse antibodies (Molecular Probes, Inc.). After several washes with PBS, the sections were coverslipped with antifade mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Immunoelectron Labeling with TSP-1

Samples of tissues obtained from the U.S. Eye Bank were fixed in 2.5% paraformaldehyde in phosphate buffer (pH 7.2), washed three times in phosphate buffer containing 0.1 M glycine, and dehydrated through a graded series of ethanol (50%, 70%, 80%, and 90% ethanol solutions, 20 minutes at each concentration; n = 3, mean age, 37 ± 16.5 years). The samples were then transferred to resin (London Resin White; TAAB Laboratories, Aldermaston, UK) for infiltration, embedded in molds containing fresh resin, and polymerized at 50°C for 24 hours. Ultrathin sections were cut on an ultramicrotome (Ultracut E; Reichert, Vienna, Austria) and collected on gilded copper grids (G400; Agar Scientific, Stansted, UK).

We labeled for the primary mouse monoclonal antibody by first placing the tissue-bearing grids in droplets of 0.1 M glycine in PBS (×2, 10 minutes each) and then incubating them in droplets of normal goat serum (20 minutes at RT). After the excess goat serum was removed, the grids were incubated overnight at 4°C with the primary antibody (catalog no. BA18, dilution, ×50; Oncogene) in PBS buffer (pH 7.4) containing 1% BSA and 1% Tween-20 (buffer 1). In the control experiments, the primary antibody was replaced with nonspecific antibody.

The grids were washed three times for 8 minutes each in five droplets of buffer 1 and then five times (8 minutes each) in distilled water. They were subsequently transferred to the appropriate secondary antibody (1:50 dilution in PBS; pH 8.2) containing 1% BSA, 1% Tween-20, 1% normal goat serum, 1% fish gelatin, and 2% sodium chloride (buffer 2). Secondary goat anti-rabbit IgG or goat anti-mouse IgG gold-conjugated (5 nm) antibodies (British Biocell International, Cardiff, UK) were used to visualize the primary antibodies. The samples were incubated for 3 hours at room temperature, and the grids were washed three times for 8 minutes each in five droplets of buffer 2 and then five times (8 minutes each) in distilled water. After the final wash, the sections were counterstained in aqueous uranyl acetate for 1 hour and examined under a transmission electron microscope (JEM 1010I; JEOL, Tokyo, Japan).

RESULTS

iAFLP Analysis

We compared the gene expression patterns of 36 major angiogenesis-related factors in human CE and CJE by using the iAFLP

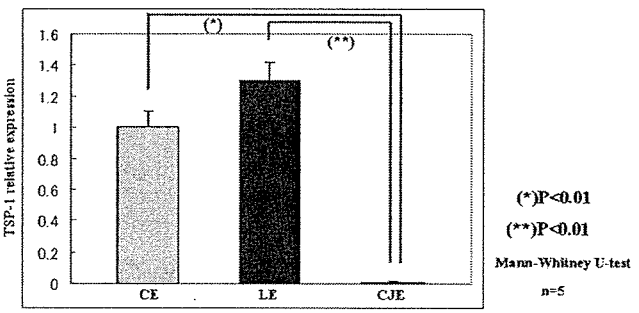

FIGURE 2. TSP-1 mRNA expression levels in the human CE, LE, and CJE, as determined by real-time PCR normalized to β-actin. The expression level of TSP-1 is significantly higher in the CE and LE than the CJE (n = 5, mean ± 0.08 and ± 0.13, respectively). The level of TSP-1 mRNA was slightly higher in the LE than the CE, but the difference was not statistically significant.

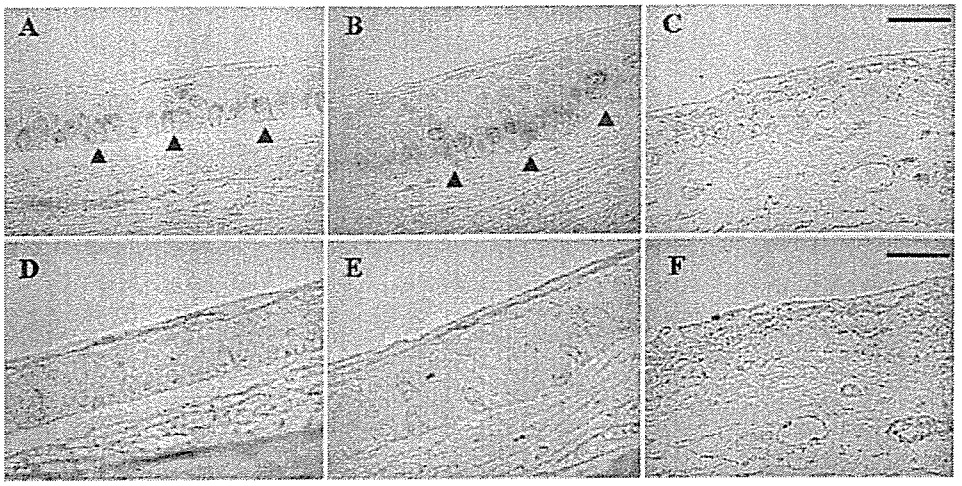

FIGURE 3. In situ hybridization of human TSP-1-specific mRNA in normal human ocular surface epithelium. The spatial anatomic distribution of TSP-1 transcripts was investigated in the CE (A), LE (B), and CJE (C). TSP-1 was expressed in corneal and limbal basal epithelial cells (A, B, *black arrowheads*). We did not detect TSP-1 transcripts in the CJE (C). (D–F) Sense RNA probes showed negligible levels of reactivity. Scale bars, 50 μm.

method. Of the 36 primers tested, 18 yielded products of the expected sizes, suggesting that the signals represented concentrations of target transcripts.

Among 36 factors, only TSP-1 was significantly upregulated in the CE (Fig. 1, yellow box). When we examined the expression pattern of keratins and ribosomal protein subunits used for validation, we found significant upregulation of CK3 and -12 in the CE, and of CK4 and -13 in the CJE and found similar expression patterns of 19 ribosomal protein subunits (Fig. 1). These results support the validity of the method used.

Real-Time PCR for TSP-1

The expression of TSP-1 mRNA in the CE, LE, and CJE ($n = 5$) was determined by real-time PCR. Compared with the CJE, mean TSP-1 mRNA was significantly upregulated in the CE and LE ($P < 0.01$, Mann-Whitney test). TSP-1 mRNA was slightly higher in the LE than the CE, although the difference was not statistically significant (Fig. 2).

In Situ Hybridization

In situ hybridization revealed the spatially restricted distribution of TSP-1 transcripts in the CE and LE, particularly in the basal epithelial cells (Figs. 3A, 3B, black arrowheads). We did not detect TSP-1 transcripts in any of the CJE layers (Fig. 3C). Sense RNA probes showed negligible levels of reactivity in all samples (Figs. 3D–F).

Immunohistochemistry for TSP-1

There was clear evidence of TSP-1 on the basement membrane side of the CE (Fig. 4A). Some patchy staining was detected in fibrous tissues and the corneal stroma below the LE (Fig. 4C, white arrows). Although we could detect moderate staining in the sclera distant from CJE, no staining was found in and just below the CJE (Fig. 4A). The area of TSP-1 expression in the ocular surface epithelium corresponded with Bowman's layer (Figs. 4A, 4C).

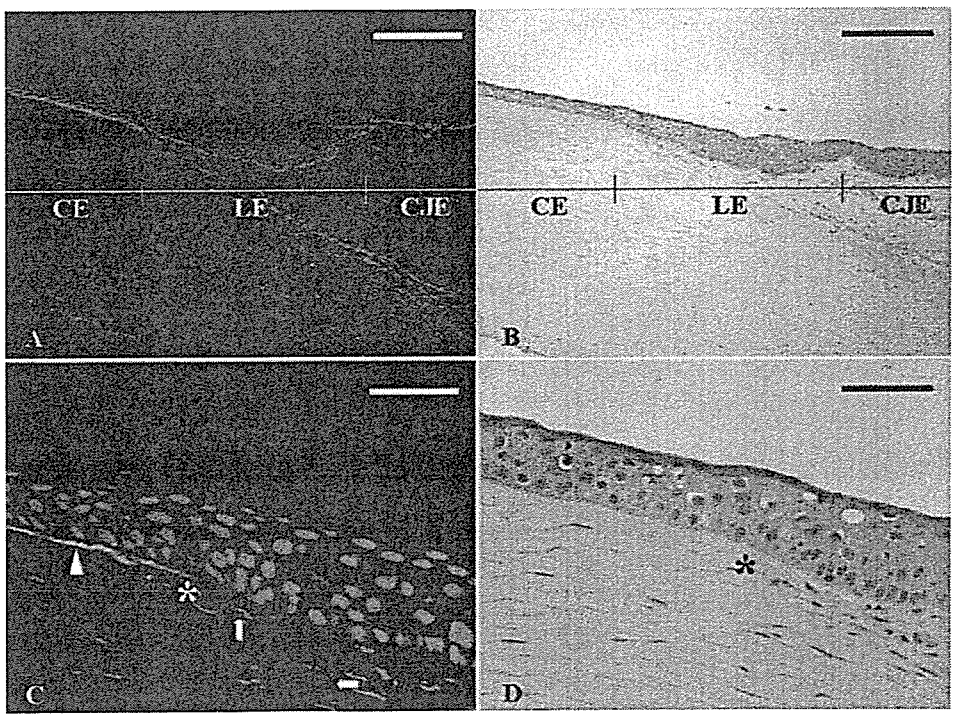

FIGURE 4. Representative immunohistochemical staining for TSP-1 (A) and hematoxylin-eosin staining in human ocular surface epithelium (CE, LE, and CJE; B). The enlarged micrographs show the area of transition between the CE and LE (C, D; *) termination of Bowman's layer. TSP-1 was present on the basal membrane side of the CE (A). Its distribution coincided with the area of Bowman's layer (C, *arrowhead*). Some patchy staining was detected in fibrous tissues and the corneal stroma below the LE (C, *arrows*). Although we detected moderate staining in the sclera distant from CJE, no staining was found in and just below the CJE (A). Scale bars: (A, B) 200 μm; (C, D) 50 μm.

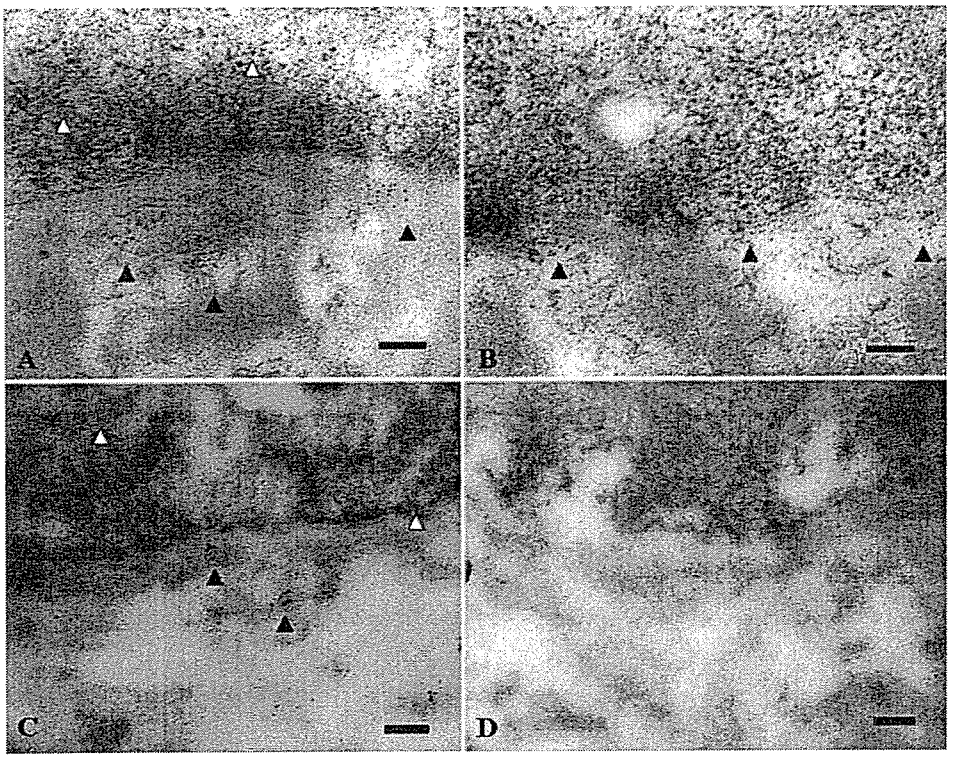

FIGURE 5. Transmission electron micrographs of the basal region of the CE (A, B), the LE (C), and the CJE (D). Significant labeling for TSP-1 was found in Bowman's layer (A, *black arrowheads*), inside basal cells (A, *white arrowheads*), and in the basal cell membrane (B, *black arrowheads*). There was some labeling just below the LE (C, *black arrowheads*) and inside basal cells (C, *white arrowheads*), but little or no labeling in the basal region of the CJE (D). The 5-nm gold particles appear as *black dots*. Scale bars, 100 nm.

Immunoelectron-Labeling with TSP-1

In the CE, Bowman's layer was strongly labeled for TSP-1 (Fig. 5A, black arrowheads). TSP-1 labeling was also found in the basal region of the basal cells (Fig. 5A, white arrowheads) and occasionally in the basal membrane of the epithelial cells (Fig. 5B, black arrowheads). In the LE, there was slight labeling just below the epithelial cells (Fig. 5C, white arrowheads), in the basal region of the basal cells, and occasionally in the basal membrane of the epithelial cells (Fig. 5C, black arrowheads). In contrast, the basal region of the CJE exhibited little or no labeling (Fig. 5D).

DISCUSSION

The cornea is one of the few avascular tissues in the adult body. Its avascularity is essential for corneal transparency and visual acuity. The mechanisms that maintain corneal avascularity and the characteristic angiogenesis-related differences between the cornea and conjunctiva remain poorly understood. Ours is the first report of the gene-expression patterns of major angiogenesis-related factors in the CE and CJE. We found that among 36 angiogenesis-related factors, TSP-1, one of the major antiangiogenic factors, was expressed at significantly higher levels in the CE.

TSP-1 was first identified as a platelet protein secreted on platelet activation[17,18] with a role in platelet aggregation.[18,19] TSP-1 has been described as a multifunctional protein that affects cell adhesion, migration, and proliferation[20,21] and plays a role in angiogenesis by exerting inhibitory effects on corneal neovascularization.[5,22,23] In mice, a deficit in a single antiangiogenic factor did not result in spontaneous corneal neovascularization.[24,25] We postulate that human corneal avascularity is also regulated by multiple antiangiogenic factors rather than a single one. Of interest, TSP-1, one of the major antiangiogenic factors contributing to corneal avascularity[5,6] was expressed at significantly higher levels in the human CE than in the CJE.

Our observation that TSP-1 transcripts were detected primarily in the basal cells of the CE and LE and that the protein was present in and just below the basal cells of both epithelia led us to postulate that TSP-1 was synthesized by basal corneal and limbal epithelial cells and that it was secreted primarily in the basal direction. TSP-1 is secreted by many types of cells including fibroblasts,[26,27] vascular endothelial cells,[26,27] smooth muscle cells,[26] macrophages, and monocytes.[28] Ours is the first documentation of its secretion in human ocular surface epithelium. In the current study, we provide evidence that in the human CE and LE, TSP-1 is secreted by basal cells, mainly toward the basal side.

Our immunoelectron microscopic study disclosed the detailed distribution of TSP-1 in the CE. Hiscott et al.[29] detected TSP-1 in the basal epithelial cell cytoplasm and basement membrane, but not within Bowman's layer. The presence of TSP-1 at the cell–matrix interface is not unexpected, because there is evidence that TSP-1 exists both as a secreted protein and as an insoluble extracellular matrix molecule[30,31] that binds to several macromolecules, such as heparin[32] and fibronectin,[33] and that it acts as an integrator of cell–matrix interactions.[34] We detected high levels of TSP-1 in Bowman's layer. TSP-1 has a high affinity for some collagens, particularly collagen type V,[35,36] and the main constituents of Bowman's layer are collagens I, III, and V.[37,38] We postulate that this explains its presence in Bowman's layer. The function of TSP-1 as an adaptor and modulator of cell–matrix interactions results in tissue remodeling and accelerated wound healing[20,21] and the transformation of latent TGF-β1 into an active form[39] that prompts the migration of corneal epithelial cells. Thus, TSP-1 in Bowman's layer may contribute to corneal epithelial cell migration and corneal avascularity. Studies are under way in our laboratory to elucidate the physiological implications of the observed TSP-1 distribution.