Table 1
Primer pairs for β-actin, keratin 12, and the p63 isoforms

	Gene/p63 isoform	Left primer	Right primer	Product size (bp)
	Beta actin	GGACTTCGAGCAAGAGATGG	ATCTGCTGGAAGGTGGACAG	402
	Keratin 12	AAGGTGATGGTTTGGAGGAA	AATCATGGGGCAGATCTTGT	199
Isoform-determining regions	TA domain	TCGTAGAAACCCCAGCTCAT	TTGTTTGTCGCACCATCTTC	199
	ΔNp specific region	CTGGAAAACAATGCCCAGAC	TGGAGAGAGAGCATCGAAGG	192
	α specific region	AGGGGCTGACCACCATCTAT	GTCTCACTGGAGCCCACACT	196
	α,β sharing region	CCACAGATTGCAGCATTGTC	GTGAATCGCACAGCATCAAT	304(α), 210(β)
	γ specific region	CCCGGAGAGAAACTCCAAA	TTGGGTCTCTGAGCCAAAGT	211
Full-length p63 isoforms	TAp63α	GAAGATGGTGCGACAAACAA	ATGATGAACAGCCCAACCTC	1436
	TAp63α,β	GAAGATGGTGCGACAAACAA	ATCGCATGTCGAAATTGCTC	1547(α), 1453(β)
	TAp63γ	GAAGATGGTGCGACAAACAA	TTCCTGAAGCAGGCTGAAAG	1130
	ΔNp63α	CTGGAAAACAATGCCCAGAC	ATGATGAACAGCCCAACCTC	1390
	ΔNp63α,β	CTGGAAAACAATGCCCAGAC	ATCGCATGTCGAAATTGCTC	1499(α), 1405(β)
	ΔNp63γ	CTGGAAAACAATGCCCAGAC	TTCCTGAAGCAGGCTGAAAG	1082

Primer pairs listed above were designed to detect the five isoform-determining regions and the six p63 isoforms.

random oligoprobe (biotin-5′-NNNNNNNNNNNNNNNNNNNN) was used as a negative control. Electrophoresed PCR products were transferred to a nylon membrane (Hybond N+, Amersham Biosciences, NJ, USA) using a standard upward capillary transfer method. The membrane was alkaline-fixed, dried and incubated in a hybridization buffer containing 0.5 M Church-phosphate buffer, 1 mM EDTA and 7% SDS. After pre-hybridization at 65°C for 5 min, the oligonucleotide probe was added to the buffer to a final concentration of 1 ng/ml. Then the membrane was incubated at 45°C for 16 hr. After a wash at 45°C, the membrane was incubated in 0.1 M maleic acid buffer containing 0.15 M NaCl, 1× blocking solution (Roche Diagnostics, Indianapolis, IN, USA) and streptavidin–alkaline phosphatase (×6000, Roche Diagnostics). After washing, chemiluminescent substrate (CDP-Star, Roche Diagnostics) was applied to the membrane to detect the signals, and images were taken using a chilled CCD camera (VersaDoc 5000; Bio-Rad Laboratories, Hercules, CA, USA). All oligonucleotides were synthesized and purchased from Promega.

3. Results

Of the 27 samples that were obtained using the laser microdissection device, 25 were successfully amplified with the Super SMART PCR cDNA synthesis kit, exhibiting moderately strong smearing, ranging from 0.4 to 3.0 kb, as shown in Fig. 3. The RTase-omitted sample did not exhibit such smearing (data not shown), indicating that contaminated genomic DNA was not amplified by the above procedure. Keratin 12 was strongly expressed in all layers of the corneal epithelial cells and in the superficial to intermediate layer of the limbal epithelial cells. The limbal basal cells exhibited only a faint band for keratin 12, which demonstrated enriched keratin 12 negative cells. All samples exhibited positive PCR bands for the β-actin gene, demonstrating successful reverse transcription and cDNA amplification.

For p63-isoforms, we designed primer pairs to detect five isoform-determining regions (TA domain, ΔNp specific region, α specific region, β specific region, γ specific region) and six p63-isoforms (TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ) (Fig. 2 and Table 1). The TA

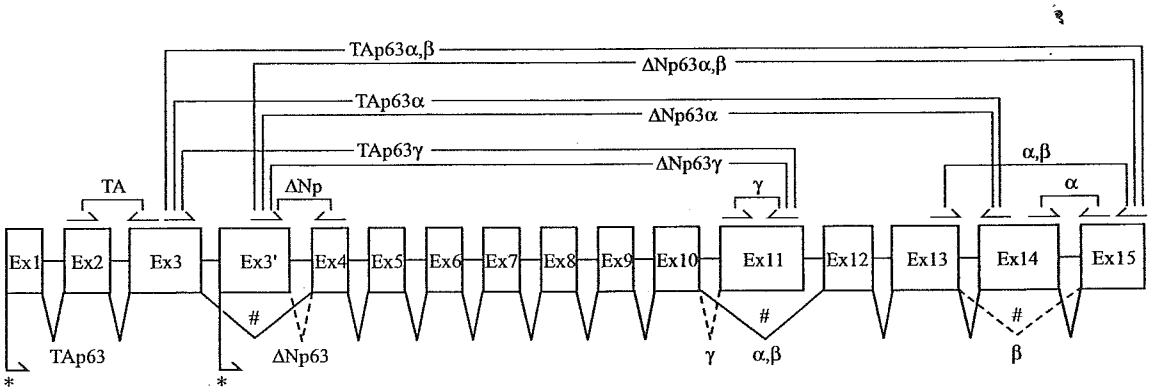

Fig. 2. Schematic diagram of the p63 genomic structure. The genomic structure of the *p63* gene is depicted according to the UCSC Genome Bioinformatics site (http://genome.ucsc.edu/). Exons are represented by boxes and introns by horizontal bars. The upper half represents the position of each primer pair. The lower half represents an alternative transcription initiation (*) and alternative splicing pattern (#) for each transcript variant. For clarity, some primer positions are not strictly indicated.

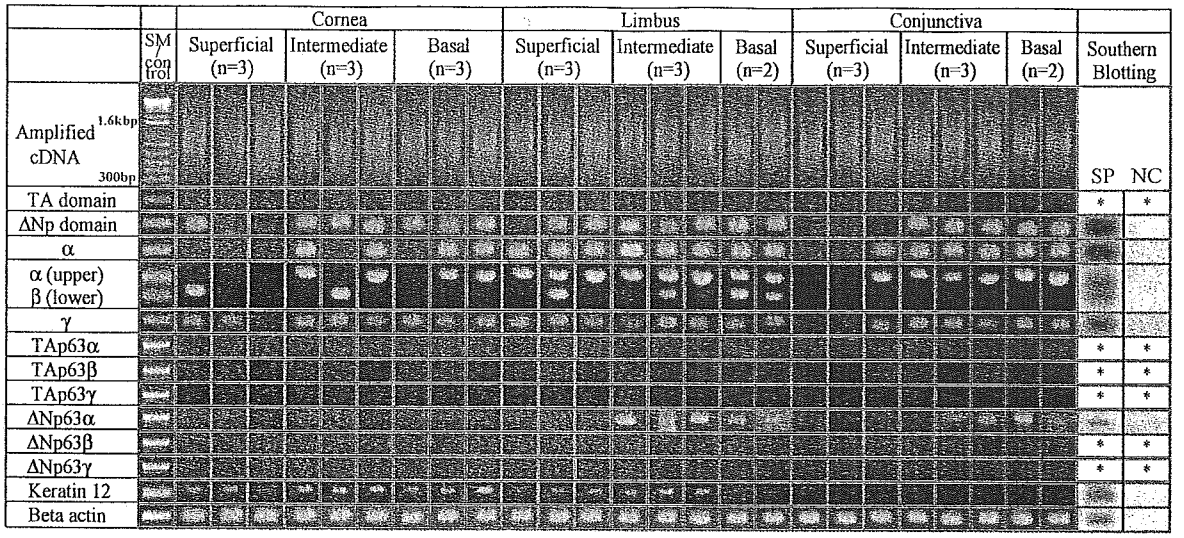

Fig. 3. Results of RT-PCR analysis. Results of RT-PCR for isoform-determining regions and individual p63-isoforms and those for keratin 12 and β-actin are shown. For the primer pair to detect isoform-determining regions of both α and β, the upper band corresponds to α and the lower band to β. Note that not all layers of the conjunctiva from all donors demonstrate a positive band for keratin 12, and limbal basal samples from all donors demonstrate only a faint band for keratin 12; which may imply successful enrichment of the keratin 12 negative, putative limbal stem cells by the laser micro-dissection device. The left-hand column (SM/control) shows the size markers and results of positive controls for all the primer pairs. Representative results of Southern blotting are also demonstrated in the right-hand columns (SP, specific probe; NC, negative control). For primer pairs without a positive band from any layers of any epithelia, Southern blot analysis was omitted (*).

domain was not detected in any layers of any epithelia (Figs. 3 and 4). The ΔNp specific region was detected in the basal to intermediate layers of all types of epithelia and in the superficial layer of the limbus and the cornea (Figs. 3 and 4). The α-isoform specific region was detected in all layers of the conjunctiva and the limbus and was also detected in the basal to intermediate layers of the cornea (Figs. 3 and 4). As there is no specific region for the β-isoform, a primer pair flanking the α-isoform specific region (exon 14) was designed to detect both the α- and β-isoforms with a different product length of 304 or 210, respectively (Fig. 2 and Table 1). The corresponding PCR

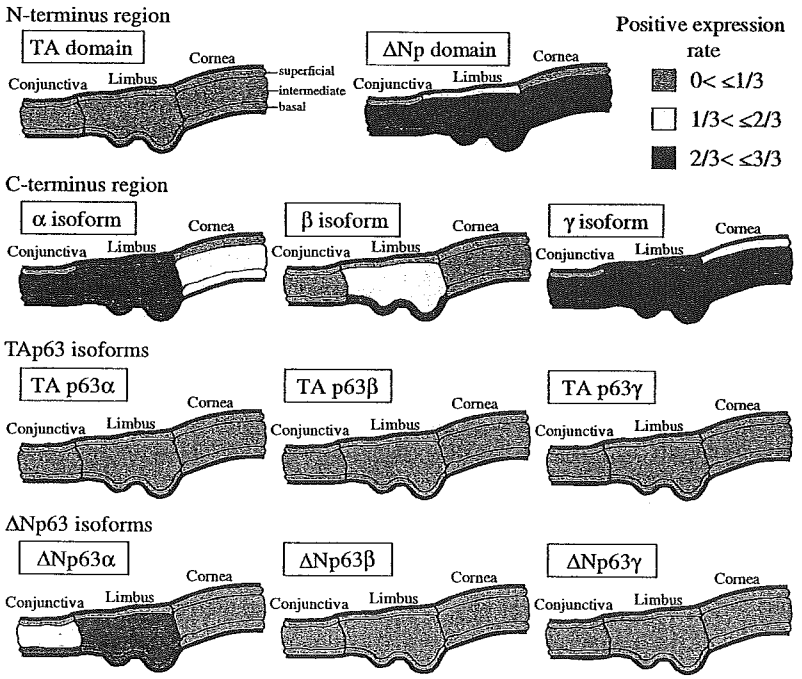

Fig. 4. Schematic representation of the spatial distribution of the five isoform-determining regions and the six p63 isoforms based on the RT-PCR results. The positive expression rate for each primer pair is summarized and depicted as colour-coded schemes for each primer pair. Each colour is represented as follows: 0 < green ≤ 1/3; 1/3 < yellow ≤ 2/3; 2/3 < red ≤ 3/3.

bands for the β-isoform were detected in the basal to intermediate layers of the limbus (Figs. 3 and 4). All conjunctival samples exhibited no positive band for this isoform. The γ-isoform specific region was detected in almost all layers of all epithelia (Figs. 3 and 4). The TAp63 isoforms, including TAp63α, TAp63β, and TAp63γ, were not detected in any layer of any epithelia (Figs. 3 and 4). ΔNp63α was detected in the basal to intermediate layers of the limbus and conjunctiva, whereas no layer of the cornea exhibited a positive band for this isoform (Figs. 3 and 4). The ΔNp63β and ΔNp63γ isoforms were not detected in any layers of any epithelia (Figs. 3 and 4). It was confirmed that all primer pairs were functional using the positive control plasmids. Southern blot analysis confirmed the authenticity of all of the positively detected RT-PCR products (Fig. 3).

4. Discussion

In the present study, we investigated the spatial distribution of p63 isoforms within the various layers of the ocular surface epithelia. Among the six p63 isoforms expressed within the ocular surface epithelia, the ΔNp63α isoform seems to be the major transcript, especially in the basal to intermediate layers of the limbal and conjunctival epithelia. Such a tissue distribution pattern in the limbus and cornea is quite consistent with the previous report by Pellegrini et al. (2001). Also, such a tissue distribution pattern in the conjunctival epithelium is consistent with a previous report (Sakoonwatanyoo et al., 2004). Accordingly, the positive signals recognized as p63 in these previous immunostaining experiments seem to be the ΔNp63α isoform. Similar to our present data, it has been reported that this isoform is the most highly expressed p63 isoform in epidermal keratinocytes (Yang et al., 1998). Therefore, the biological significance and function of this isoform seems to be similar in these epithelia and perhaps similar in all types of stratified epithelia.

Little is known about the functional difference and biological significance of the individual p63 isoforms. The most firmly established and noteworthy observation so far is the antipode functions between the TAp63 and ΔNp63 isoform groups. The TAp63 group is required for or elicits several p53-dependent cellular processes, including cell cycle arrest or apoptosis (Flores et al., 2002) via its N-terminal TA domain. On the other hand, the ΔNp63 group lacks this domain, thus yielding a dominant-negative effect against such cellular processes and ultimately achieving the maintenance of cell proliferation (Yang et al., 1998; Liefer et al., 2000). Interestingly, Koster et al. (2004) reported a significant role of the TAp63 isoforms in initiating epidermal stratification during the embryonic stage. Our present data indicating greater expression of the ΔNp63 specific region than the TAp63 specific region in the basal layer seems to be quite consistent with the generally accepted notion that the basal layer has more proliferative cells than other layers. In addition, although it is still controversial whether p63 is really a specific marker for corneal epithelial stem cells, this protein may contribute, at least in part, to maintenance of the putative corneal and/or conjunctival stem cells via such anti-apoptotic activity.

While the functional difference determined by the N-terminal region is well established, as described above, much less is known about the functional divergence of the three C-terminal isotypes. The α-isoform is known to have a transactivation inhibitory domain (TID) and a sterile-α-motif (SAM) domain in the C-terminal region. It has been reported that the TID domain has both *cis*- and *trans*-inhibitory activity for the TAp63α and the TAp63β/γ protein, respectively (Yang et al., 1998; Ozaki et al., 1999). It is well established that the SAM domain is involved in protein–protein interaction through homo- or hetero-oligomerization with other proteins that also have this domain (Schultz et al., 1997). Thus, the predominantly expressed ΔNp63α within ocular surface epithelia suggests several complicated but as yet unknown interactions via these functional domains. How, why and when ocular surface epithelial cells use a specific ΔNp isoform is the next matter to be resolved.

We found discordance between the results of the isoform-determining regions and those of the six p63-isoforms. Some samples showed positive PCR bands for ΔNp and α, β and/or γ, but did not exhibit any of the combinations ΔNp63α, ΔNp63β, or ΔNp63γ. The sensitivity of the PCR may explain this discrepancy. In general, amplification efficiency is higher in short fragments than in long fragments. The length of the PCR products was approximately 200–300 bases for the five isoform-determining regions and approximately 1000–1500 bases for the six p63-isoforms. Accordingly, PCR amplification of the six p63 isoforms appears to be less efficient than that of the five isoform-determining regions. Probably, ΔNp63β and ΔNp63γ are expressed in the ocular surface epithelia, perhaps much less than ΔNp63α. Other types of isoform-sensitive expression analyses, such as Northern hybridization or RNase protection assays, would provide meaningful information regarding this. However, such experiments seem to be quite difficult because these procedures generally require a much greater amount of RNA than can be obtained by laser micro-dissection.

In conclusion, ΔNp63α appears to be the most dominant isoform within the ocular surface epithelia, especially in basal to intermediate layers of the limbus and the conjunctiva. The biological functions of this isoform may be to retain the proliferative capacity of these cells via a dominant negative effect against the p53-dependent cell cycle arrest or apoptosis. Identification of the upstream regulators and downstream effectors of this protein should be the next goal to expand our understanding of how cell proliferation and differentiation are regulated within the ocular surface epithelia.

Acknowledgements

We gratefully thank all staff at the North–West Lion EyeBank Foundation, especially Monty Montoya, Bernie Iliakis, Doug Marcoux, Malcom An and Jeremy Shuman, for kindly helping us to obtain fresh corneal tissues for investigation in this study. We also thank Joanna J. Connon for proofreading of this paper.

This work was supported by grants (15791001, 16390502) from the Japanese Ministry of Education, Science, Culture and Sports, and Ministry of Health, Labour and Welfare. This work was also supported by a research fund from the Kyoto Foundation for the promotion of medical science.

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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

Accepted for publication Aug 9, 2005.

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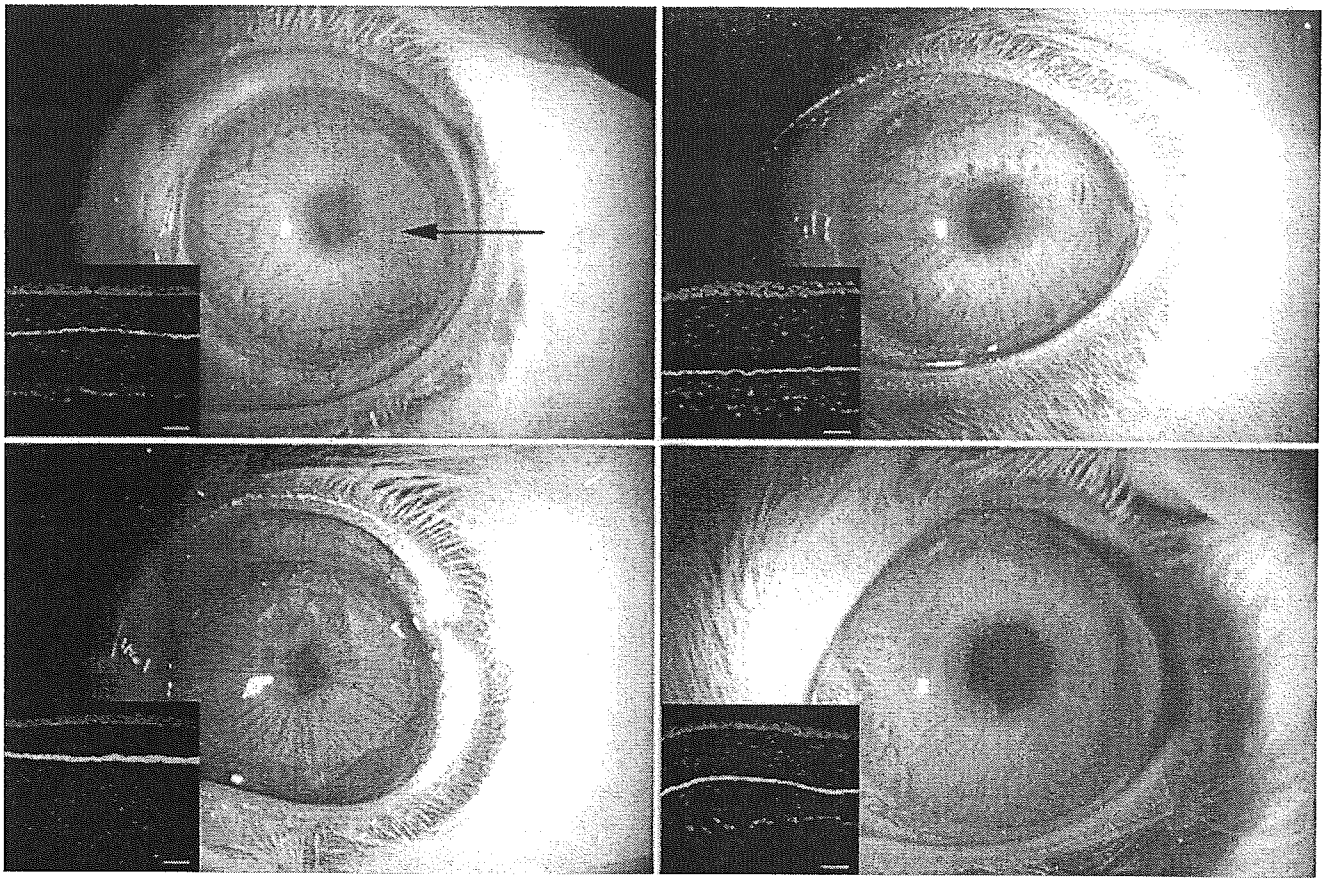

FIGURE 1. Transplanted amniotic membrane in corneal stroma with photographs of the right rabbit eyes before enucleation. (Top left) the transplanted amniotic membrane was still visible at 1 month (arrow). Visibility improved progressively at 3 (top right), 7 (bottom left), and 10 months (bottom right). 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-

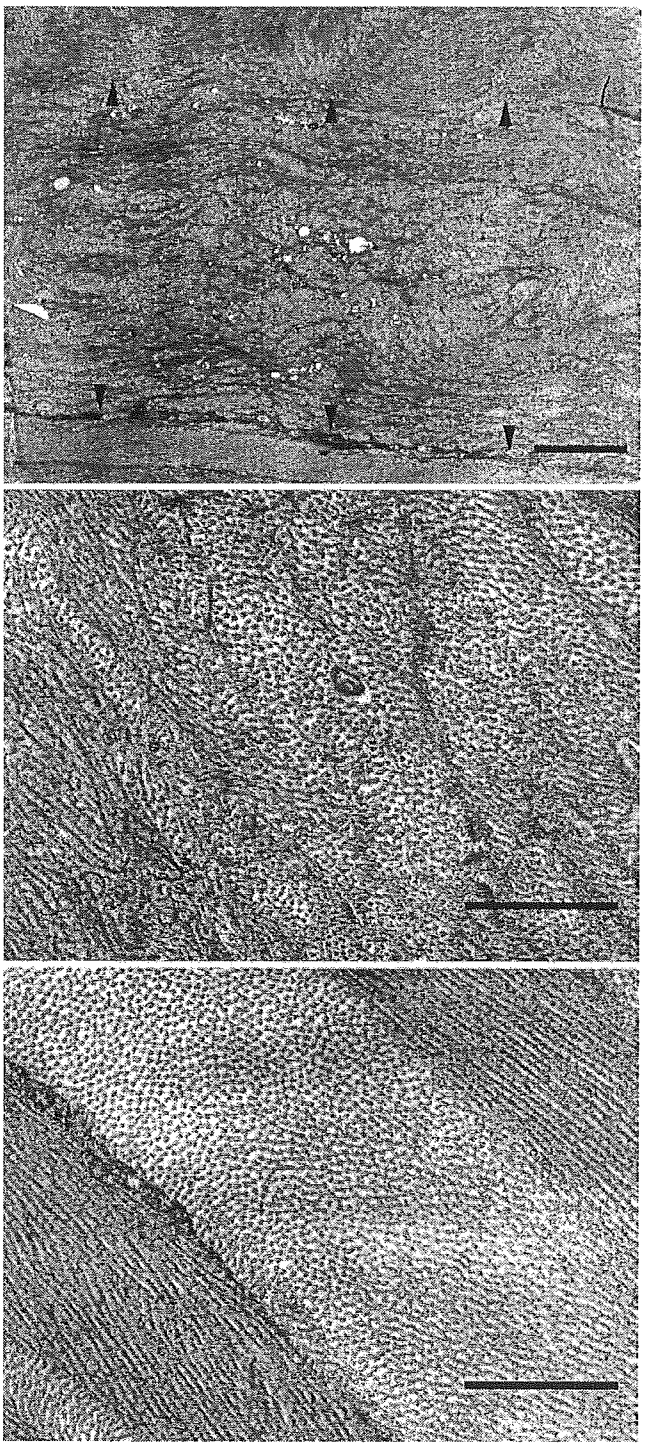

FIGURE 2. Transmission electron microscopy of the amniotic membrane and corneal stroma. (Top) No obvious change to the implanted amniotic membrane ultrastructure was observed at 10 months (arrowheads denote inside edge of amniotic membrane). Scale bar = 3 μm. (Middle) High magnification image of disorganized 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

All financial and material support for this research was met jointly by Japanese Society for the Promotion of Science and The Royal Society. The design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript was undertaken independently of the funding organizations.

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The Use of Autologous Serum in the Development of Corneal and Oral Epithelial Equivalents in Patients with Stevens-Johnson Syndrome

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Purpose. To evaluate the use of autologous serum (AS) from patients with severe ocular surface disease (OSD) in the development of transplantable corneal and oral epithelial tissue equivalents and to compare it with the use of conventional culture methods by using fetal bovine serum (FBS).

Methods. AS was obtained from patients with severe OSD secondary to Stevens-Johnson syndrome. Corneal and oral epithelial cells were cultivated in medium supplemented with either AS or FBS. Corneal and oral epithelial equivalents were constructed on denuded amniotic membranes. The bromodeoxyuridine (BrdU) ELISA cell proliferation assay and colony-forming efficiency (CFE) of cells cultivated in AS- or FBS-supplemented media were compared. The morphologic characteristics and the basement membrane assembly of cultivated epithelial equivalents were analyzed by light and electron microscopy, as well as by immunohistochemistry.

Results. BrdU proliferation assay and CFE analysis showed that human corneal and oral epithelial cells cultivated in AS-supplemented media had comparable proliferative capacities compared with FBS-supplemented media. The corneal and oral epithelial equivalents cultivated in AS- and FBS-supplemented media were morphologically similar and demonstrated the normal expression of tissue-specific keratins and basement membrane assembly. The presence of a well-formed stratified epithelium, a basement membrane, and hemidesmosomal attachments was confirmed by electron microscopy.

Conclusions. AS-supplemented cultures were effective in supporting the proliferation of human corneal and oral epithelial cells, as well as the development of transplantable epithelial equivalents. The use of AS is of clinical importance in the development of autologous xenobiotic-free bioengineered ocular surface equivalents for clinical transplantation. (*Invest Ophthalmol Vis Sci.* 2006;47:909–916) DOI:10.1167/iovs.05-1188

Severe ocular surface disease (OSD), arising from conditions such as Stevens-Johnson syndrome (SJS) and ocular cicatricial pemphigoid, is a potentially devastating condition with significant visual morbidity. In such cases, the corneal epithelial stem cells in the limbus are destroyed, resulting in invasion of the corneal surface by surrounding conjunctiva, neovascularization, chronic inflammation, ingrowth of fibrous tissue, and stromal scarring.[1–3] Conventional corneal transplantation in these patients is associated with dismal results. Alternative methods such as keratoepithelioplasty and limbal transplantation have been used to reconstruct these severely damaged eyes, with improved clinical outcomes.[4,5] More recently, cultivated corneal epithelial stem cell transplantation has demonstrated promising results and has gained general acceptance as an effective treatment modality.[6–9] We,[10] together with other investigators,[11] have also demonstrated the effective use of autologous cultivated oral epithelial transplantation for the treatment of severe OSD, with the advantage that this reduces the risk of allograft rejection and the need for long-term steroids or immunosuppression.

The currently preferred method of cultivating corneal or oral epithelial cells requires the use of xenobiotic materials, such as fetal bovine serum (FBS) and 3T3 feeder cells, in the culture system. Various serum-free culture systems, developed to obviate the need for FBS, have mainly been used to study the roles of various growth factors.[12–14] The clinical use of these serum-free culture systems has been limited because of their lower efficacy for cell propagation compared with bovine serum–supplemented medium. In the development of tissue equivalents for clinical transplantation, the ideal culture condition is one that is safe from disease transmission, as well as being able to support cell proliferation and differentiation. The use of autologous human serum as an alternative to FBS is therefore significantly advantageous, because it eliminates the need for bovine material in the culture process. This is particularly important when ex vivo expanding cells for clinical transplantation, because it reduces the risk of transmission of diseases, for example, spongiform encephalitis, or other unknown infections.

Ang et al.[15] previously showed that human serum was able to support the in vitro and in vivo proliferation of cultivated human conjunctival cells. We wanted to determine whether autologous serum (AS) from patients with severe OSD was similarly efficacious in supporting cell proliferation, as well as the development of cultivated ocular surface epithelial equivalents, compared with conventional FBS supplemented culture conditions. We also sought to show that these transplantable bioengineered epithelial equivalents bore similar morphologic characteristics and differentiation-related keratin expression as the tissue of origin and possessed the necessary cell-to-cell and cell-to-substrate junctional elements (such as integrins and hemidesmosomes) for ensuring graft integrity after transplan-

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Supported in part by Grants-in-Aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; a research grant from the Kyoto Foundation for the Promotion of Medical Science; and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

Submitted for publication September 6, 2005; revised October 27, 2005; accepted January 11, 2006.

Disclosure: **T. Nakamura**, None; **L.P.K. Ang**, None; **H. Rigby**, None; **E. Sekiyama**, None; **T. Inatomi**, None; **C. Sotozono**, None; **N.J. Fullwood**, None; **S. Kinoshita**, None

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tation. To our knowledge, a study of this nature has not been previously reported. This study has important clinical implications, because it provides the basis for developing safer autologous bioengineered tissues for clinical transplantation.

Materials and Methods

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.

Preparation of Amniotic Membrane

Human amniotic membranes (AM) were obtained from mothers who had undergone cesarean sections. Under sterile conditions, the membranes were washed with PBS that contained antibiotics (5 mL 0.5% levofloxacin) and were stored at −80°C in modified medium (Dulbecco's modified Eagle's medium; GibcoBRL, Rockville, MD) and glycerol (Wako Pure Chemical Industries, Osaka, Japan) in the ratio of 1:1 by volume. Immediately before use, the AM was thawed, washed three times with sterile PBS that contained antibiotics, and cut into pieces approximately 4 × 4 cm in size. The overlying amniotic epithelial cells were removed by incubation with 0.02% EDTA (Nacalai Tesqu Co., Kyoto, Japan) at 37°C for 2 hours, followed by gentle scraping with a cell scraper (Nunc International, Naperville, IL).

Subjects and Harvesting of Serum

Patients with severe OSD secondary to SJS were enrolled in the study. These patients manifested severe destruction of the ocular surface, limbal stem cell deficiency, total conjunctivalization of the cornea, and conjunctival cicatrization. The patients comprised 1 male and 3 females; their ages ranged from 27 to 69 years (mean, 49.3 ± 22.4 years). AS was obtained from these patients. Venesection was performed at the antecubital fossa under aseptic conditions; 30 mL of blood was collected into a sterile container, centrifuged, and filtered; the resultant serum (approximately 10 mL) was purified. Each patient's serum was stored in sterile tubes at −30°C. For experimental controls, we used 4 randomly selected distinct lots of FBS (ICN Inc., Aurora, OH).

Cultivation of Human Corneal and Oral Epithelial Cells

Corneal Epithelial Culture. Because all these patients had bilateral limbal stem cell deficiency, with the absence of any normal corneal epithelium, corneal epithelial cells were obtained from human corneoscleral rims from the Northwest Lion Eye Bank (Seattle, WA). These corneoscleral rims were first incubated at 37°C for 1 hour with 1.2 IU dispase to separate the epithelial cells, as previously described.[16] Cells from the limbal and peripheral corneal region were carefully separated from the underlying stroma.

Oral Epithelial Culture. We obtained oral mucosal biopsy specimens (2–3 mm^2) from these patients and volunteers while they were under local anesthesia. The submucosal connective tissue was removed with scissors to the extent possible; the resulting samples were then incubated at 37°C for 1 hour with 1.2 IU dispase, as previously described,[17] and were treated with 0.05% Trypsin-EDTA solution for 10 minutes at room temperature to separate the cells.

After cell separation, the resultant corneal and oral epithelial cells were then seeded onto tissue culture dishes at a density of 1×10^4 cells/cm^2. The culture medium consisted of defined keratinocyte growth medium (KGM; Amniotec, Tokyo, Japan) supplemented with 5% AS or 5% FBS, as well as insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human-recombinant epidermal growth factor (10 ng/mL), and penicillin-streptomycin (50 IU/mL).[18] Cultures were incubated at 37°C in a 5% CO_2–95% air incubator, and the medium was changed every day.

Quantitation of Proliferative Capacity and Clonal Growth of Cells

The following proliferation assays were used to assess the proliferative capacity of the cells cultured with either AS- or FBS-supplemented media.

Bromodeoxyuridine (BrdU)-ELISA Cell Proliferation Assay. The proliferative capacity of human corneal or oral epithelial cells (passage 1) was determined by a BrdU-ELISA cell proliferation assay (Amersham Biosciences, Freiburg, Germany) by using a previously reported protocol.[15,19] Analyses were performed on the sixth day of passage. Cultured cells were incubated with 10 μM BrdU-labeling solution for 20 hours at 37°C, followed by washing with 250 μL PBS that contained 10% serum per well. They were fixed with 70% ethanol in hydrochloric acid for 30 minutes at −20°C and incubated with 100 μL of monoclonal antibody against BrdU for 90 minutes, followed by 100 μL peroxidase substrate per well. The BrdU absorbance in each well was measured directly with a spectrophotometric microplate reader at a test wavelength of 450 nm and a reference wavelength of 490 nm. This gave us a measure of the degree of cell proliferation, which we termed the proliferation index (PI). Each sample was cultured in triplicate.

Colony-forming Efficiency. The clonal growth ability of cultured corneal or oral epithelial cells by using AS- and FBS-supplemented media was determined by the colony-forming efficiency (CFE). Cells were plated at a clonal density of 1000 cells onto 6-well culture dishes. A colony was defined as a group of eight or more contiguous cells.[15,19] The colonies were fixed on day 8, stained with 0.1% Truidine blue and counted independently by 3 investigators; the data were then averaged. Each sample was cultured in triplicate.

The CFE was defined as follows

$$\text{CFE (\%)} = \frac{\text{Colonies formed at the end of growth period}}{\text{Total number of viable cells seeded}} \times 100\ (\%).$$

The Development of Corneal and Oral Epithelial Equivalents

Corneal and oral epithelial cells were initially enzymatically separated as described above. The separated cells were then seeded onto denuded amniotic membranes spread on culture inserts in 6-well culture plates, at a density of 1×10^5 cells/well. These were cocultured with mitomycin-C-inactivated 3T3 fibroblasts (2×10^4 cells/cm^2).[17,18] The cells were incubated with AS- and FBS-supplemented culture media, as described above. The cultures were submerged in medium for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 1 to 2 days. Cultures were incubated at 37°C in a 5% CO_2–95% air incubator, and the medium was changed every day.

Immunohistochemistry

Immunohistochemical studies of several tissue-specific keratins and basement membrane–related proteins in corneal and oral epithelial sheets cultivated by using AS- or FBS-supplemented media were carried out by following our previously described method.[20,21] Normal human cornea and oral samples were also examined for comparison. Briefly, cryostat sections (7-μm thick) were placed on gelatin-coated slides and air-dried, then rehydrated in PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with 2% BSA at room temperature for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with the primary antibody (Table 1), then washed three times in PBS that contained 0.15% Triton

TABLE 1. Primary Antibodies and Source

Antibodies	Category	Dilution	Source
Integrin α6	Mouse monoclonal	×200	Chemicon, International, Inc. (Temecula, CA)
Integrin β4	Mouse monoclonal	×500	Chemicon, International, Inc.
Integrin β1	Mouse monoclonal	×500	Chemicon, International, Inc.
Collagen IV	Mouse monoclonal	×200	MP Biomedicals (Eschwege, Germany)
Collagen VII	Mouse monoclonal	×100	Chemicon, International, Inc.
Laminin 5	Mouse monoclonal	×100	Chemicon, International, Inc.
Keratin 3	Mouse monoclonal	×50	Progen (Wieblingen, Germany)
Keratin 4	Mouse monoclonal	×200	Novocastra (New Castle upon Tyne, UK)
Keratin 12	Goat polyclonal	×100	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
Keratin 13	Mouse monoclonal	×200	Novocastra

X-100 for 15 minutes. Control incubations were with the appropriate normal mouse and goat IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody, and omission of the primary antibody for the respective specimen. After staining with the primary antibody, the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, fluorescein (FITC)-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR). After several washings with PBS, the sections were coverslipped by using antifading mounting medium that contained propidium iodide (Vectashield; Vector, Burlingame, CA) and were examined by confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Electron Microscopy

Human donor corneal epithelial cells cultured on denuded amniotic membrane by using AS- or FBS-supplemented media were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Specimens were fixed in 2.5% glutaraldehyde in 0.1M PBS, washed three times for 15 minutes in PBS and post-fixed for 2 hours in 2% aqueous osmium tetroxide. They were then washed three more times in PBS before being passed through a graded ethanol series (50, 70, 80, 90, 95, and 100%). For SEM preparation, specimens were transferred to hexamethyldisilazane (TAAB Laboratories Equipment Ltd., Berkshire, UK) for 10 minutes and allowed to air-dry. When dry, the specimens were mounted on aluminum stubs and sputter-coated with gold before examination in a digital SEM (JEOL JSM 5600; Herts, UK). For TEM preparation, the specimens were embedded in epoxy resin (Agar 100-epoxy resin; Agar Scientific, Essex, UK). Ultrathin (70 nm) sections were collected on copper grids and stained for 1 hour with uranyl acetate and 1% phosphotungstic acid, then for 20 minutes with Reynold's lead citrate before examination on a TEM (JEOL JEM 1010).

RESULTS

Proliferative Capacity and Clonal Growth

In both AS- and FBS-supplemented media, human corneal and oral epithelial cells formed colonies with ovoid and round cells, with some elongated cells (Fig. 1). The epithelial morphology of cells cultivated in AS- and FBS-supplemented media was comparatively similar. BrdU proliferation assay showed that the PIs of human corneal epithelium cultivated by using AS and FBS were 3.00 ± 0.16 and 3.10 ± 0.03, respectively (Fig. 2A). These differences were not statistically significant. The PIs of human oral epithelium cultivated by using AS and FBS were 2.50 ± 0.31 and 2.67 ± 0.16, respectively (Fig. 2A). These differences were also not statistically significant.

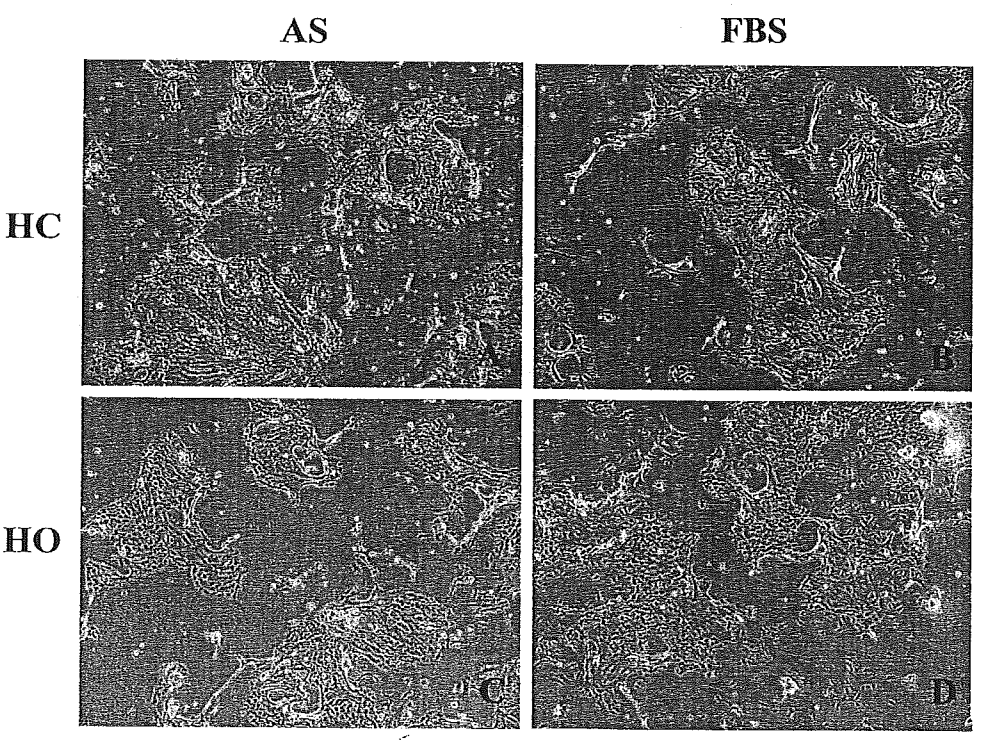

FIGURE 1. Representative phase contrast appearance of human corneal epithelial cells (A, B) and human oral epithelial cells (C, D) cultivated in AS- (A, C) and FBS-supplemented (B, D) media on day 6. In both AS- and FBS-supplemented media, human corneal and oral epithelial cells formed colonies consisting of ovoid and round cells with some elongated cells. The epithelial cell morphology was very similar between the 2 groups. Original magnification, ×40. HC, human corneal epithelium; HO, human oral epithelium.

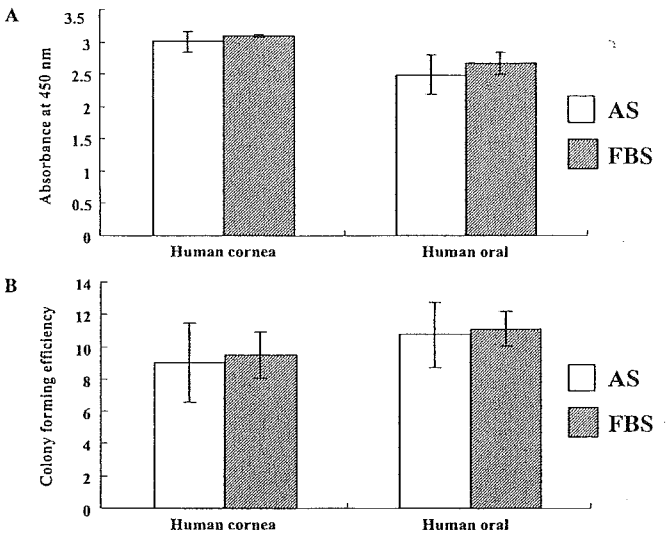

FIGURE 2. (A) BrdU ELISA cell proliferation assay of corneal and oral epithelial cells cultivated in AS- (n = 12) and FBS-supplemented (n = 12) media. The bars show the mean values of BrdU absorbance in each culture condition. Proliferation indices (PI) of human corneal epithelial cells were 3.00 ± 0.16 (AS) and 3.10 ± 0.03 (FBS), whereas PIs of human oral epithelial cells were 2.50 ± 0.31 (AS) and 2.67 ± 0.16 (FBS). There were no statistically significant differences between them. (B) Colony forming efficiencies (CFE) of human corneal epithelial cells were 9.0 ± 2.45% (AS) and 9.5 ± 1.45% (FBS), whereas CFEs of human oral epithelial cells were 10.75 ± 2.01% (AS) and 11.1 ± 1.05% (FBS). There were also no statistically significant differences between them.

The CFEs of human corneal epithelial cells were 9.0 ± 2.45% (AS) and 9.5 ± 1.45% (FBS), whereas the CFEs of human oral epithelial cells were 10.75 ± 2.01% (AS) and 11.1 ± 1.05% (FBS) (Fig. 2B). For both corneal and oral epithelial cells, there were no statistically significant differences between the CFEs of AS- and FBS-supplemented cultures.

Differentiation of Cultivated Corneal and Oral Epithelial Cells

The expression patterns of several tissue-specific keratins in cultivated corneal (Fig. 3) and oral (Fig. 4) epithelium were investigated immunohistochemically. Negative control sections, incubated with normal mouse and goat IgG, and primary antibody omission exhibited no discernible specific immunoreactivity over the entire region. The immunoreactivity observed in each specimen was compared with these controls.

In the normal (Figs. 3A3, 3B3) and cultivated (Figs. 3A1, 3A2, 3B1, 3B2) corneal epithelial cells, the cornea-specific keratins 3 and 12 were expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal cell layers. The expression patterns of these keratins were similar between epithelial sheet cultivated by using AS (Figs. 3A1, 3B1) and FBS (Figs. 3A2, 3B2).

In human normal oral epithelium, keratin 3 (Fig. 4A3) and keratin 13 (Fig. 4C3) were expressed in all epithelial layers except basal cell layers; keratin 4 was expressed in the superficial and upper half of intermediate layers (Fig. 4B3). In the cultivated epithelial sheet, keratins 3 and 13 were expressed in almost all epithelial cell layers (Figs. 4A1, 4A2, 4C1, 4C2), whereas keratin 4 was sporadically expressed in the superficial cell layers (Figs. 4B1, 4B2). The expression pattern of these keratins was also similar between epithelial sheets cultivated when using AS (Figs. 4A1, 4B1, 4C1) and FBS (Figs. 4A2, 4B2, 4C2).

Basement Membrane Assembly Protein Expression

Immunohistochemistry showed linearly positive staining of integrin α6 (Figs. 5A1–5A3, Figs. 6A1–6A3), integrin β4 (Figs. 5B1–5B3, Figs. 6B1–6B3), collagen IV (Fig. 5D13, Figs. 6D1–6D3), collagen VII (Figs. 5E1–5E3, Figs. 6E1–6E3), and laminin 5 (Figs. 5F1–5F3, Figs. 6F1–6F3) on the basement membrane side of corneal and oral epithelial cells. In contrast, integrin β1 was expressed in the cell membrane of epithelial cells (Figs. 5C1–5C3, Figs. 6C1–6C3). These AS- and FBS-derived epithelial sheets maintained the phenotypic characteristics of normal in vivo corneal and oral epithelia.

Electron Microscopy

SEM examination revealed a continuous layer of flat squamous polygonal epithelial cells in corneal epithelial cells cultivated by using AS (Fig. 7A1) and FBS (Fig. 7B1). The cells in both groups were closely attached to each other, with tightly opposed cell junctions and distinct cell boundaries, and the apical surface of the cells was covered with numerous microvilli (Figs. 7A2, 7B2).

TEM examination of the corneal epithelial culture sheet showed that the cells appeared healthy and had differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (Figs. 7A3, 7A4, 7B3, 7B4). The basal

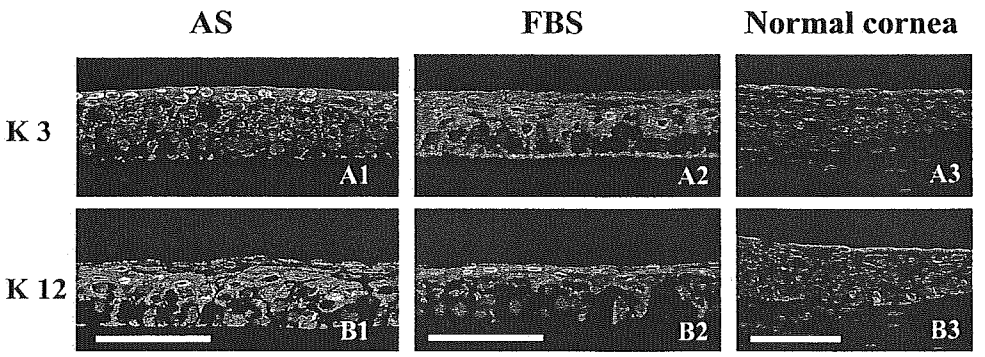

FIGURE 3. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (A1, B1) and FBS-supplemented (A2, B2) media, compared with normal in vivo cornea epithelium (A3, B3). In all 3 epithelia, cornea-specific keratins 3 (A1–A3) and 12 (B1–B3) were expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal-cell layers. The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS- and FBS-supplemented culture systems. Scale bars, 100 μm.

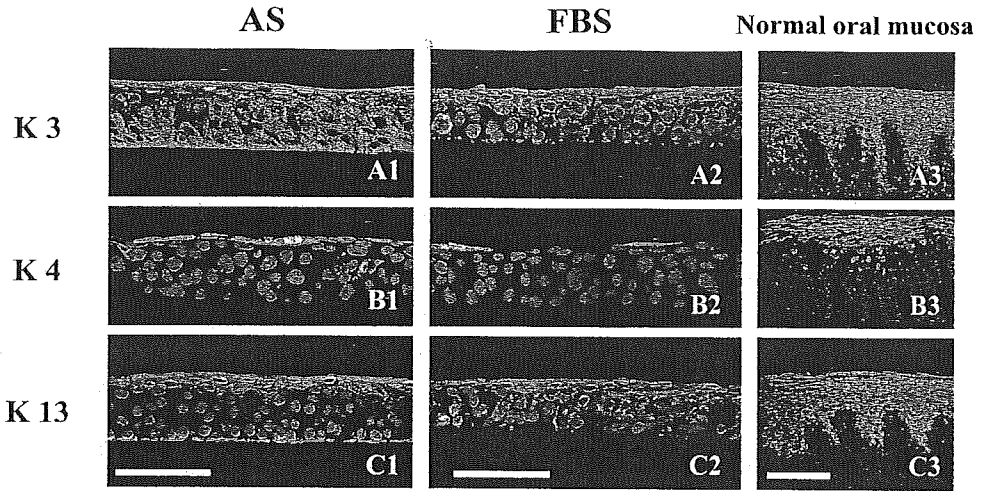

FIGURE 4. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (A1–C1) and FBS-supplemented (A2–C2) media, compared with normal in vivo oral epithelium (A3–C3). In normal oral epithelium, keratin 3 (A3) and keratin 13 (C3) were expressed in all epithelial layers, except the basal-cell layers, and keratin 4 was expressed in the superficial and upper half of the intermediate layer (B3). In the cultivated epithelial sheet, keratins 3 and 13 were expressed in almost all epithelial cell layers (A1, A2, C1, C2), whereas keratin 4 was sporadically expressed in the superficial cell layers (B1, B2). The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS- and FBS-supplemented culture systems. Scale bars, 100 μm.

epithelial cells adhered well to the AM substrate with hemidesmosome attachments, and produced basement membrane material (Figs. 7A5, 7B5). In all cell layers, the epithelial cells were comparatively closely attached to neighboring cells by numerous desmosomal junctions (Figs. 7A6, 7B6). Morphologic patterns were similar between AS- and FBS-culture systems.

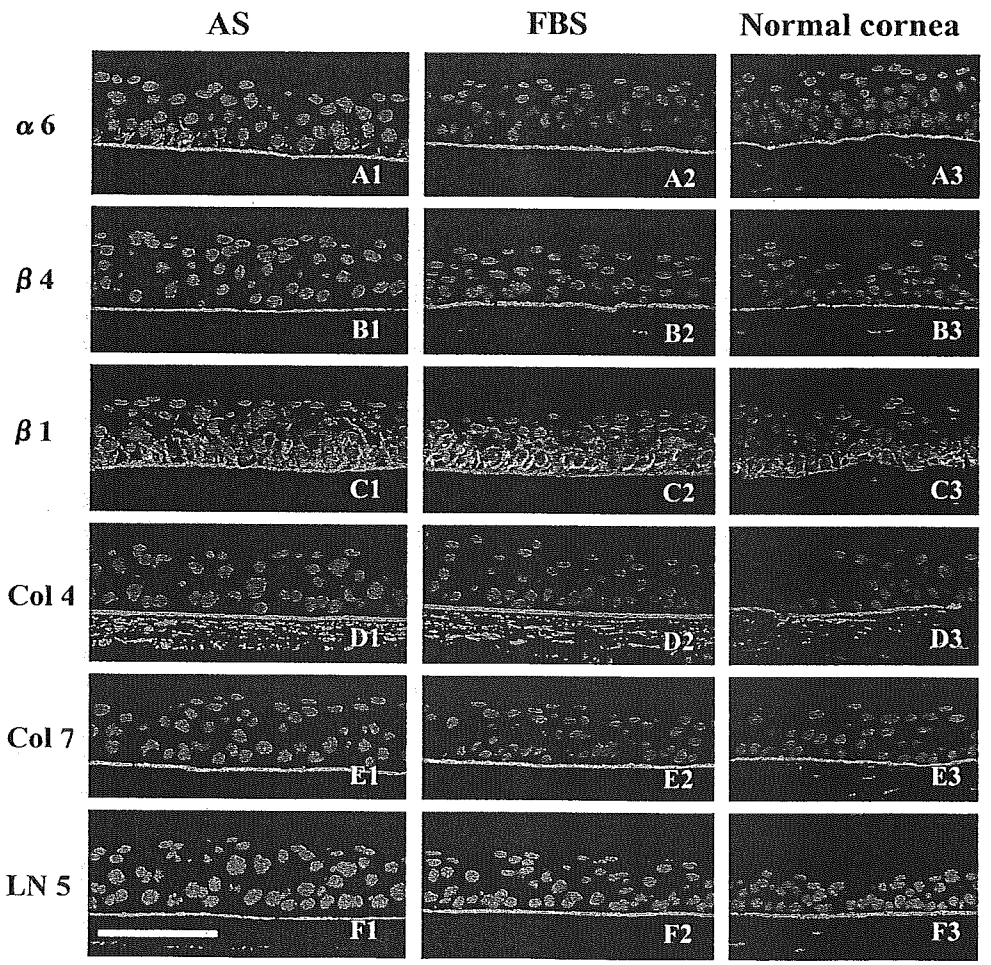

FIGURE 5. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (A1–F1) and FBS-supplemented (A2–F2) media, compared with normal in vivo cornea epithelium (A3–F3). There was linear positive staining of integrin α6 (A1–A3), integrin β4 (B1–B3), collagen IV (D1–D3), collagen VII (E1–E3), laminin 5 (F1–F3) on the basement membrane side of cultivated corneal epithelial cells, similar to that of normal corneal epithelium. In contrast, integrin β1 was expressed in the cell membrane of epithelial cells (C1–C3). The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS- and FBS-supplemented culture systems. Scale bars, 100 μm.

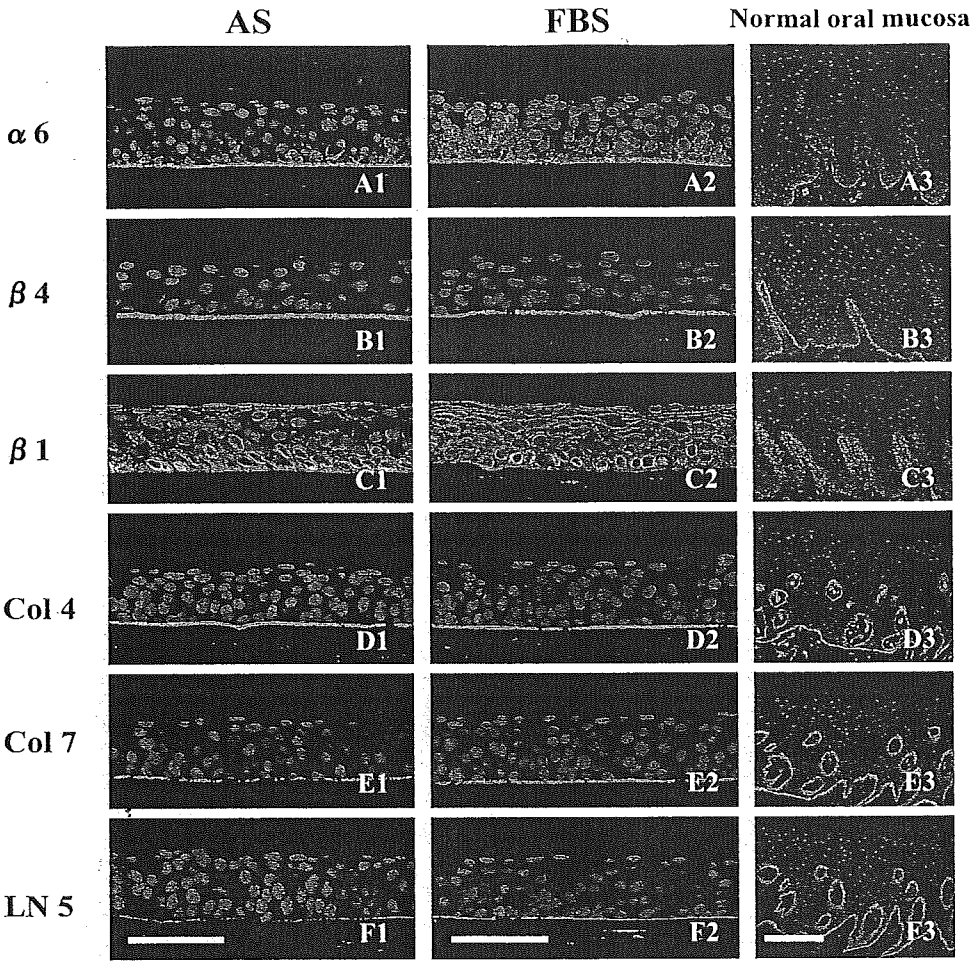

FIGURE 6. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (A1–F1) and FBS-supplemented (A2–F2) media, compared with normal in vivo oral epithelium (A3–F3). There was linear positive staining of integrin α6 (A1–A3), integrin β4 (B1–B3), collagen IV (D1–D3), collagen VII (E1–E3), laminin 5 (F1–F3) on the basement membrane side of cultivated oral epithelial cells, similar to that of normal oral epithelium. In contrast, integrin β1 was expressed in the cell membrane of epithelial cells (C1–C3). The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS- and FBS-supplemented culture systems. Scale bars, 100 μm.

DISCUSSION

Previous studies on cultivated ocular tissue equivalents have relied primarily on bovine serum-supplemented media.[6–8] However, the use of FBS in the culture system is a major concern, because BSE cannot be detected by any known in vitro assay. Cultivated ocular surface epithelial transplantation has mainly been used for treating various severe OSDs where conventional therapy has had limited success. As such, the use of AS for the development of bioengineered ocular surface equivalents would be of particular clinical relevance in these patients. We demonstrate for the first time that AS-supplemented media derived from patients with SJS were able to support epithelial-cell propagation, as well as the development of tissue-equivalents bearing similar morphologic and ultrastructural characteristics as the normal in vivo tissues.

Previous reports on epithelial equivalents have mainly focused on obtaining differentiated, stratified tissue equivalents.[22–24] However, the ability of culture media to support the proliferation of cells is a critical issue in propagating cells for clinical transplantation, if these cells are to continue to regenerate the tissue of origin.[19,25] It has previously been demonstrated that cells cultivated by using human serum from normal patients supported the in vitro and in vivo proliferation of human conjunctival epithelial cells.[15] SJS is a major cause of severe OSD, and afflicted patients often have multisystemic involvement. In our study, we addressed the critical issue of whether AS-supplemented media from these SJS patients were able to support in vitro cell proliferation as effectively as conventional bovine serum-supplemented media. By using BrdU-ELISA proliferation assays, as well as clonal growth studies, we showed that human corneal and oral epithelial cells cultivated in AS-supplemented media had in vitro capacities comparable with those of conventional FBS supplemented media. These findings are important in supporting the use of AS for the ex vivo expansion of epithelial cells.

We further demonstrated that these AS-derived cultivated corneal and oral epithelial cells formed confluent stratified epithelial sheets on AM. The histologic appearance of these epithelial sheets closely resembled the tissue of origin in terms of cell morphology, as well as degree of stratification. Ultrastructural examination of the epithelial equivalents cultivated in AS- and FBS-supplemented media revealed the presence of well-formed, multilayered epithelial sheets with tightly opposed cell junctions. The apical surface of the cultivated oral epithelial cells was covered with numerous microvilli, which was almost identical with that found in in vivo corneal epithelium. In both AS- and FBS-supplemented culture systems, cultivated corneal and oral epithelial cells each retained their innate phenotypic characteristics, as confirmed by their expression of tissue-specific keratins. These findings demonstrate the ability of AS-supplemented culture media to support the continued proliferation and differentiation of cultivated cells in bioengineered tissue equivalents, which is of paramount importance when considering its use in clinical transplantation.

A critical issue regarding the use of cultivated epithelial sheets for ocular surface reconstruction is the ability of these tissue equivalents to retain their structural integrity after transplantation. This is dependent on basal-cell attachments to the underlying substrate, as well as cell-to-cell adhesion structures.

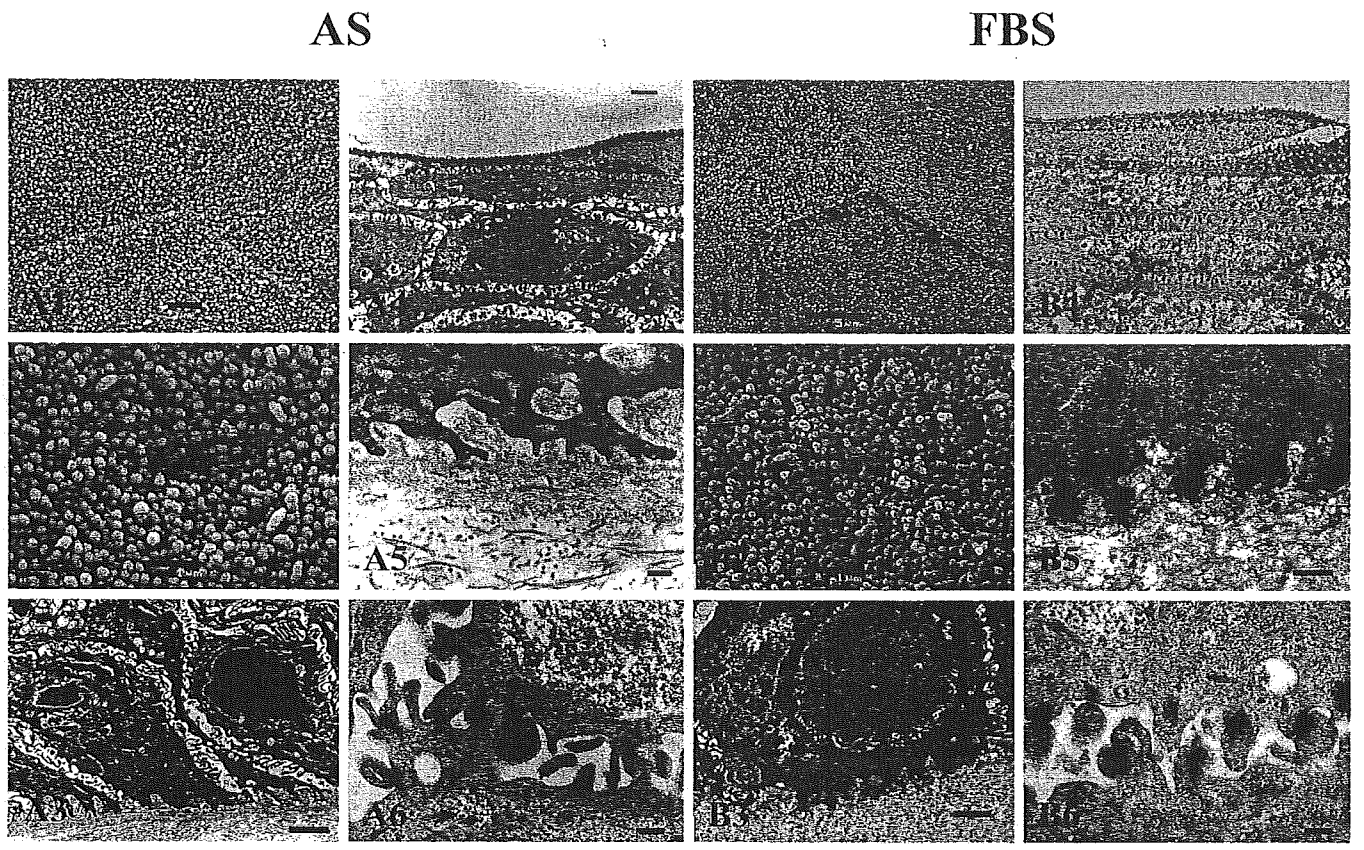

FIGURE 7. Scanning electron microscopic examination showed a continuous layer of flat squamous polygonal epithelial cells in the cultivated corneal epithelial cells when using AS (**A1**) and FBS (**B1**). These cells were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries. The apical surfaces of the AS- and FBS-derived epithelial sheets were covered with numerous microvilli (**A2**, **B2**). Transmission electron microscopic examination of the corneal epithelial culture sheet showed that the cells appeared healthy and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (**A3**, **A4**, **B3**, **B4**). The basal epithelial cells adhered well to the AM substrate with hemidesmosomal attachments and produced basement membrane material (**A5**, **B5**). In all cell layers, the epithelial cells were comparatively closely attached to neighboring cells by numerous desmosomal junctions (**A6**, **B6**). The morphologic appearance was very similar in the AS- and FBS-supplemented culture systems. Scale bars: (**B1**) 5 μm; (**A1**, **A3**, **A4**, **B3**, **B4**) 2 μm; (**A2**, **B2**) 1 μm; (**A5**, **B5**) 500 nm; (**A6**, **B6**) 200 nm.

Normal epithelial cells have specialized junctions on their cell surfaces to ensure firm adhesion to neighboring cells and the extracellular matrix below.[26-28] Desmosomal junctions are present between the cell-to-cell surfaces and give the cell-sheet structural integrity, while hemidesmosomes present on the basal-cell surfaces serve to attach the basal cells to the basement membrane.[26-28] In both AS- and FBS-supplemented cultures, TEM demonstrated the presence of a basal lamina with hemidesmosomal attachments at the basal-cell–substrate junctional zone. Immunohistochemistry confirmed the expression of α6 and β4 integrins, which are associated with hemidesmosomes, as well as the presence of collagen IV and laminin 5. These are essential for cell-to-substrate adhesion and the maintenance of tissue integrity. Adjacent cells in the cultivated sheet were also joined by numerous desmosomal junctions. These findings are important in ensuring graft integrity during surgical manipulation, as well as after transplantation.

In summary, our study is the first to demonstrate the effective use of AS from patients with SJS in supporting the ex vivo expansion of corneal and oral epithelial cells. The elimination of animal and nonhuman material from the culture system offers significant advantages over existing bovine serum–supplemented culture procedures, because it reduces the risk of transmission of zoonotic infection. These findings bring us one step closer to the development of a safe and effective xenobiotic-free bioengineered tissue equivalent for clinical transplantation. This has significant clinical implications, because these cultivated ocular surface epithelial equivalents may potentially be used in the treatment of patients with severe OSD.

Acknowledgments

The authors thank Narisato Kanamura and Takashi Amemiya for performing the oral biopsies, Hideo Honjyo for providing AM, and Hisayo Sogabe and Tomoko Horikiri for assisting with the culture procedures.

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Current Concepts and Challenges in Ocular Surface Reconstruction Using Cultivated Mucosal Epithelial Transplantation

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Abstract: Ocular surface disorders wherein corneal stem cells are deficient result in total conjunctivalization leading to severe visual dysfunction and blindness. Treatment of these diseases requires reconstruction of the ocular surface using epithelial transplantation to provide a smooth refractive corneal surface and biologic and physical barriers against a variety of destructive factors. Recently, regenerative medicine focusing on tissue-engineered techniques has been developed and established as a new clinical field. In particular, cultivated mucosal epithelial transplantation using well-differentiated, stratified epithelial sheets on amniotic membrane allows a rapid re-epithelial cover over the entire corneal surface, resulting in early reduction of inflammation and cicatrization. This surgical approach dramatically improves the prognosis of severe ocular surface diseases, especially severely inflamed corneal stem cell deficiency. This new approach not only provides early epithelialization but also allows reconstruction of the corneal surface using autologous cultivated epithelium including the cornea and oral mucosa from a small number of cell sources after amplification. Tissue-engineered strategies using autologous corneal or oral mucosal epithelial sheet transplantation avoid the risk of rejection and complications associated with immunosuppressive treatments. In addition to new cellular transplantation, combined automated lamellar therapeutic keratoplasty provides better refractive stromal interference and contributes to performance and safety of cataract surgery in severe corneal opacity cases. Although long-term prognosis and cell biologic behavior of tissue-engineered epithelium after transplantation should be carefully monitored, these recent surgical treatments have opened a new field for ocular surface reconstruction.

Key Words: cultivated mucosal epithelial transplantation, cultivated oral mucosal epithelial transplantation, ocular surface reconstruction, tissue-engineered cell sheets

(*Cornea* 2005;24(Suppl. 1):S32–S38)

The development of a variety of surgical procedures and surgical instruments has been attempted to improve outcomes and overcome severe ocular surface diseases. Because corneal tissue comprises the corneal epithelium, stroma, and corneal endothelium, component surgery is a trend that aims to retain maximal unaffected tissue to minimize surgical damage and immunologic response.[1] Recently, several major innovations have focused on these issues. New surgical technology and instruments such as microkeratome and laser technology have been developed resulting in better wound adaptation and precise excision of corneal stroma, which are essential for the improvement of visual quality. Microkeratome-assisted surgical procedures, such as automated lamellar therapeutic keratoplasty (ALTK),[2] provide lamellar dissection at the desired thickness to replace lamellar components, minimizing interface wound disturbance. The clinical success of component corneal surgery has been much improved following the development of technology. Moreover, application of biomaterial and artificial material has been tried. Amniotic membrane (AM) is now widely used as an ideal biosubstrate for the basement membrane, not only as a surgical material for pterygium surgery and ocular surface reconstruction[3] but also as a cellular substrate for tissue engineering.[4] In addition to cryopreserved AM, we have recently reported successful manipulation of sterilized and freeze-dried AM for improved convenience and medical safety.[5] Another novel field is a new surgical concept based on regenerative medicine. Ocular surface reconstruction is a pioneer frontier in the clinical practice using tissue-engineered regenerative material.[6–8] The introduction of amplification and regeneration of tissue structure from a small source of original stem cells in vitro has dramatically changed surgical procedures and expanded the options of treatment of severe ocular surface disorders. In this report, we review our current concepts and clinical application in ocular surface reconstruction using cultivated mucosal epithelial transplantation.

NEW CONCEPTS IN CULTIVATED MUCOSAL EPITHELIAL SHEET TRANSPLANTATION

The ocular surface consists of nonkeratinized epithelia: corneal and conjunctival epithelium. It is widely accepted that basal cells located in the limbal region have unlimited cell migration as a stem cell character in the cornea.[9] On the basis of this observation, limbal transplantation is a conventional

Received for publication July 5, 2005.
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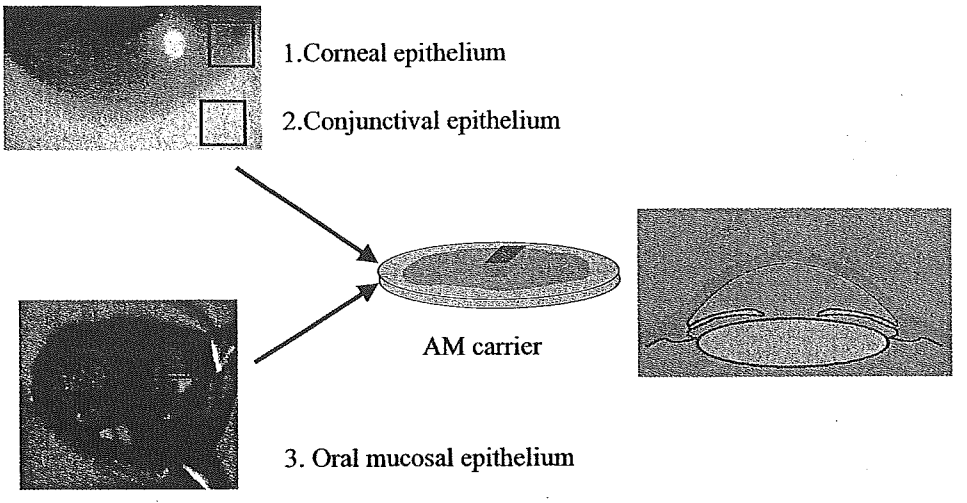

FIGURE 1. Schematic showing possible choice of cell sources, corneal and conjunctival epithelium, and oral mucosal epithelium as the nonocular origin, for cultivated mucosal epithelial sheet transplantation. Autologous or allogeneic graft sheets are applied depending on the condition of the eyes, the primary cause of disease and the patient's age and history.

surgical procedure using donor corneal explants to create new corneal epithelial cell outgrowth and resurface the ocular surface in patients with stem cell deficiency.[10] The basics of tissue-engineered regenerative medicine in corneal surgery were introduced by Pellegrini and colleagues,[6] who demonstrated the clinical application of cultivated epithelial transplantation in 1997. Several different groups have also developed tissue-engineered procedures using different cell culture techniques and carriers for the same purpose, ie, to develop the ideal clinical application.[6–8,11,12] One of the advantages of cultivation of epithelium in vitro is to amplify small cell numbers to obtain transplantable epithelial sheets without threatening the healthy unaffected eye. For successful survival of transplanted epithelium, induction of cell differentiation and stratification and safe surgical transfer are essential. The well-established fibroblast coculture system and air-lifting technique were modified to generate epithelial sheets. AM was found to be the ideal substrate and carrier for cultivated mucosal epithelial transplantation because it has a basement membrane similar to that of the cornea.[4] No biologic evidence has been reported, however, on the appropriate number of stem cells contained in the epithelial sheet to maintain the re-epithelialized surface without losing biologic characteristics.

Other critical aspects of generation of the allogeneic or autologous epithelial sheet include selection and harvest of epithelial sources. Because the ocular surface has an interface with tears, 3 different nonkeratinized, stratified epithelial sources have been assessed (Fig. 1). The corneal epithelium itself is the most suitable epithelial source for reconstruction of the corneal surface due to its original anatomic and functional characteristics. Regenerative tissue engineering can safely amplify sufficient cell numbers from small amounts of limbal tissue, which may not interfere with the future function of healthy unaffected cornea in unilateral cases.[13] Allogeneic cornea donors are also a major source of corneal epithelium for patients with bilateral ocular surface disease. Two alternative cell sources, conjunctival epithelium[14,15] and oral mucosal epithelium,[16,17] have been proposed to generate autologous mucosal epithelial sheets for the treatment of patients with bilateral ocular surface disease. Compared with corneal tissue, these 2 alternative cell types exist in highly vascularized environments. Ectopic transplantation is the only procedure for bilateral corneal stem cell deficiency. However, epithelial behavior and biologic response of adjacent tissue should be examined in future studies.

COMPARISON WITH CONVENTIONAL CORNEAL EPITHELIAL TRANSPLANTATION

Conventional corneal epithelial transplantation using explants, such as keratoepithelioplasty[18] and limbal transplantation, is also an effective procedure for the treatment of ocular surface diseases (Fig. 2). However, the success of these types of surgeries requires the process of wound healing and epithelial growth. Thus, one of the most remarkable benefits of cultivated mucosal epithelial transplantation involves the period of epithelialization. It normally takes 7–14 days to achieve epithelial wound closure by migration of epithelium for the lenticles; compared with this step, cultivated epithelial sheet transplantation provides rapid epithelialization just after transplantation, reducing epithelium-related inflammation and persistent epithelial defect. In addition to rapid wound healing, cultivated mucosal epithelial sheet transplantation using an AM carrier appears to prevent corneal scarring and lipid deposition, which are commonly observed in adjacent corneal stroma even after a postoperative period of a few years. Less scar formation of the corneal stroma by the AM carrier also provides unlimited opportunities for regrafting without visual disturbance.[19] Figure 3 demonstrates an example case of a 24-year-old man with acute phase Stevens-Johnson syndrome over a 5-year period. The patient first received a cultivated corneal epithelial transplant to treat the disorder in 1999. During the 5-year follow-up period, the patient underwent 4 cultivated mucosal epithelial transplantations following loss of transplanted epithelium resulting in conjunctivalization. After the fourth operation, the patient had a clear stroma without scarring. Although this patient had conjunctivalization due to epithelial rejection and persistent epithelial defect, the corneal stroma retained sufficient transparency and provided further regrafting opportunities and visual recovery,

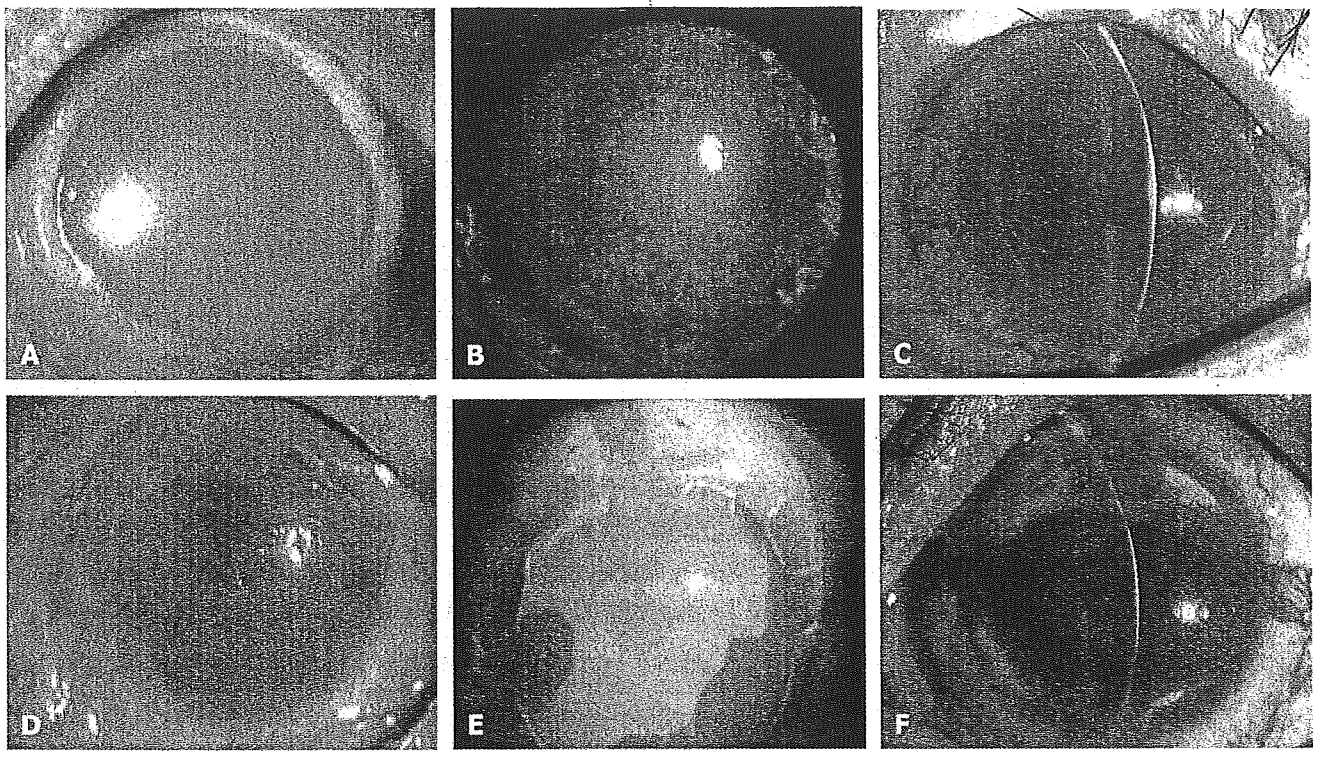

FIGURE 2. This bilateral case of a 32-year-old man in the acute phase of Stevens-Johnson syndrome demonstrated different clinical course after cultivated corneal epithelial transplantation in the right eye (A–C) and limbal transplantation in the left eye (D–F). A and D show the preoperative ocular condition with a persistent epithelial defect and corneal stem cell deficiency. Four days after transplantation, the ocular surface showed complete epithelialization (B) compared with the remaining epithelial defect on the lateral eye subject to limbal transplantation (E). Less corneal scarring with a cultivated corneal epithelial sheet on amniotic membrane was noted 2 years after transplantation (C) compared with the other eye (F).

suggesting the possible suppression of corneal scarring by the AM carrier.

SURGICAL INDICATION AND IMPLICATION FOR OCULAR SURFACE DISEASES IN ACUTE INFLAMMATORY PHASE

Indications for cultivated mucosal epithelial sheet transplantation include a variety of corneal stem cell deficiency diseases. Because this surgical procedure provides rapid epithelial cover over the entire corneal surface, patients with acute ocular surface diseases with total corneal epithelial loss such as severe chemical injury, acute phase of Stevens-Johnson syndrome, and inflamed ocular cicatricial pemphigoid are suitable subjects for this treatment compared with conventional corneal epithelial transplantation.[8] Figure 4 shows 2 typical cases of ocular cicatricial pemphigoid with massive inflammation and subconjunctival fibrosis. Cicatrization of the conjunctiva progressed due to prolonged epithelial defect and activated immunoresponse, resulting in severe dry eye with total conjunctivalization. Hence, not only rapid wound cover but also reduction and removal of subconjunctival inflammatory cells are crucial for the treatment and improvement of prognosis of these conditions. The results of the histologic examination of cicatrized tissue from the 2 cited cases are shown in Figure 5. Massive inflammatory response in the subconjunctival spaces (Fig. 5A,C) and immunoglobulin precipitation against basement membrane components (Fig. 5D) as a typical etiologic event in ocular cicatricial pemphigoid were confirmed in the resected tissue. Intraoperative mitomycin C (MMC) treatment also suppresses fibroblast proliferation and reduces inflammatory responses. AM transplantation and AM carrier of cultivated mucosal epithelial sheet may also contribute to the suppression of fibrosis and conjunctival invasion based on the same proposed mechanisms. Ocular surfaces of both cases were well reconstructed with transplanted cultivated mucosal epithelium and retained stable ocular surface during follow-up. Activity of their primary diseases was controlled without any progression of cicatrization of the conjunctiva after the proper surgical treatment at the inflamed phase. The surgical combination of cultivated mucosal epithelial transplantation and intraoperative MMC appears to be an effective tool for severe cicatrizing ocular surface disorders.

AUTOLOGOUS CULTIVATED ORAL MUCOSAL EPITHELIAL TRANSPLANTATION

In patients with bilateral limbal stem cell deficiency, autologous cultivated corneal epithelial transplantation is not an option. Finding an alternative nonocular epithelial cell source in culture is required to reconstruct by autologous cells to eliminate immunosuppressive medication. Buccal mucosal

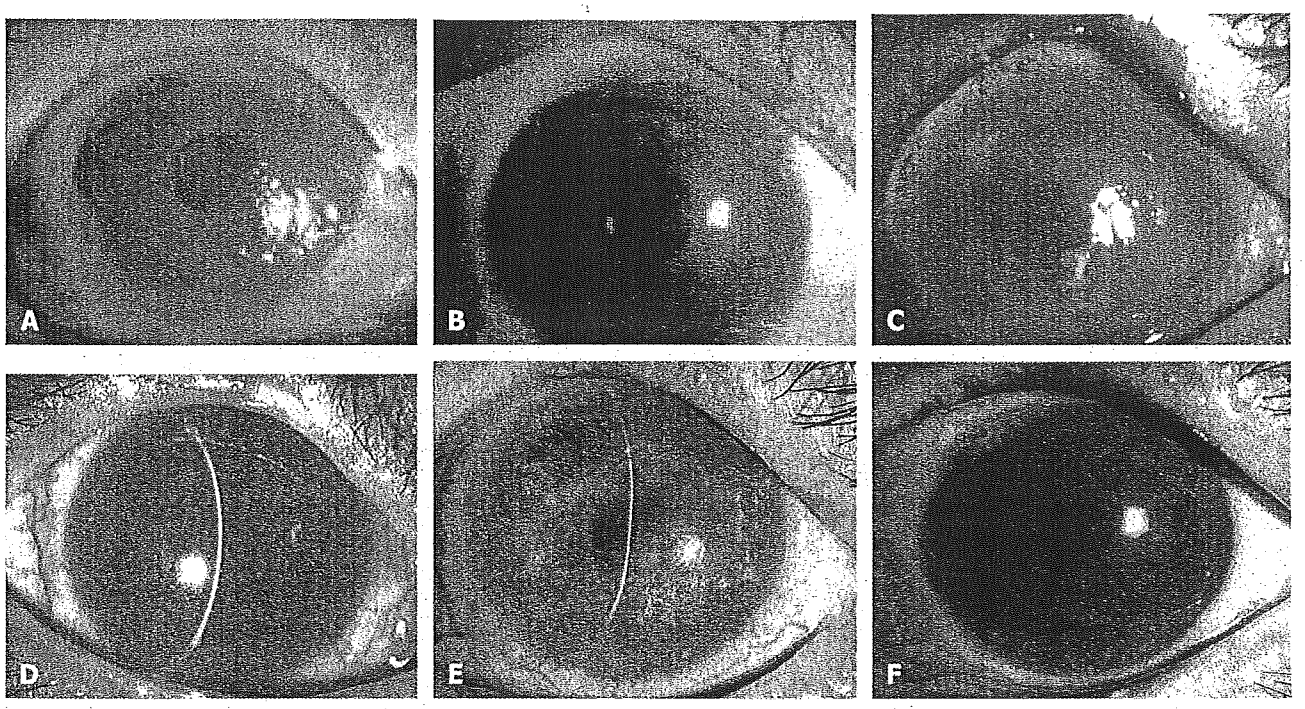

FIGURE 3. A 24-year-old patient with acute Stevens-Johnson syndrome showed successful recovery of the corneal surface at each time point cultivated corneal epithelial transplantation was conducted without stromal scarring. Preoperative condition (A); after the first operation (B); preoperative condition prior to the second operation (C); after the second operation (D); preoperative condition prior to the fourth operation (E); and recent condition after the fourth operation (F).

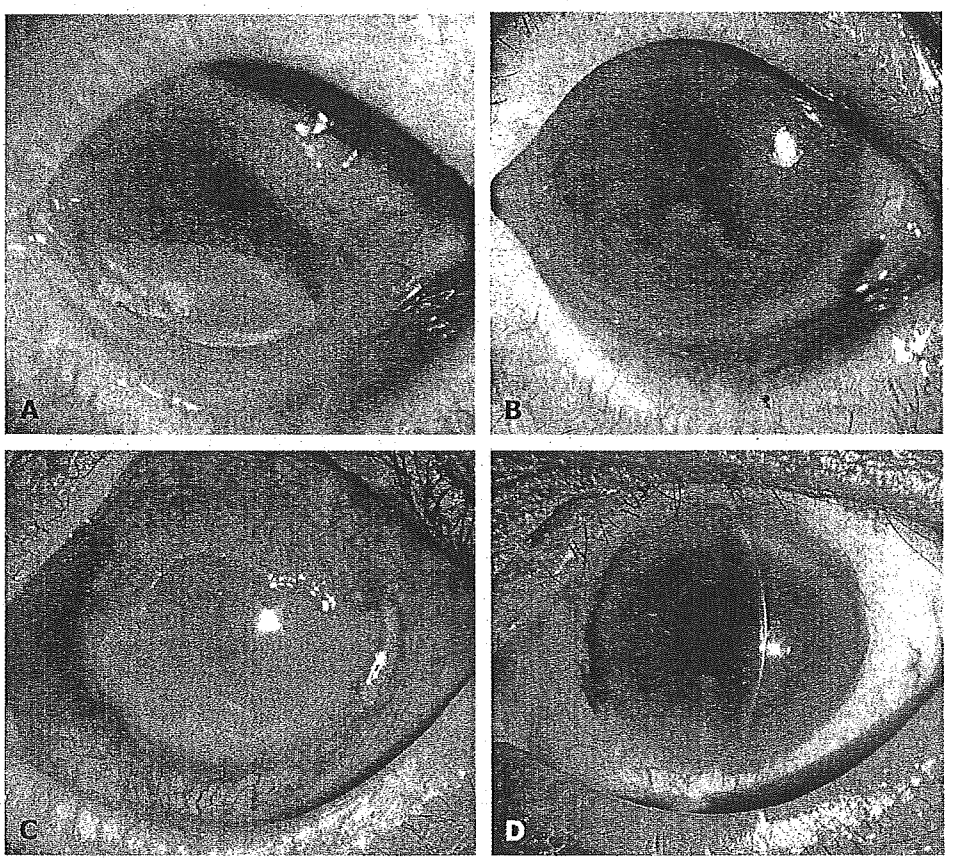

FIGURE 4. Two ocular pemphigoid cases with persistent corneal epithelial defect and ocular inflammation (A, C). Severe inflammatory response was seen surrounding the cornea. Ocular surfaces were reconstructed using cultivated corneal epithelial transplantation resulting in no epithelial defect and reduction of inflammation (B, D).

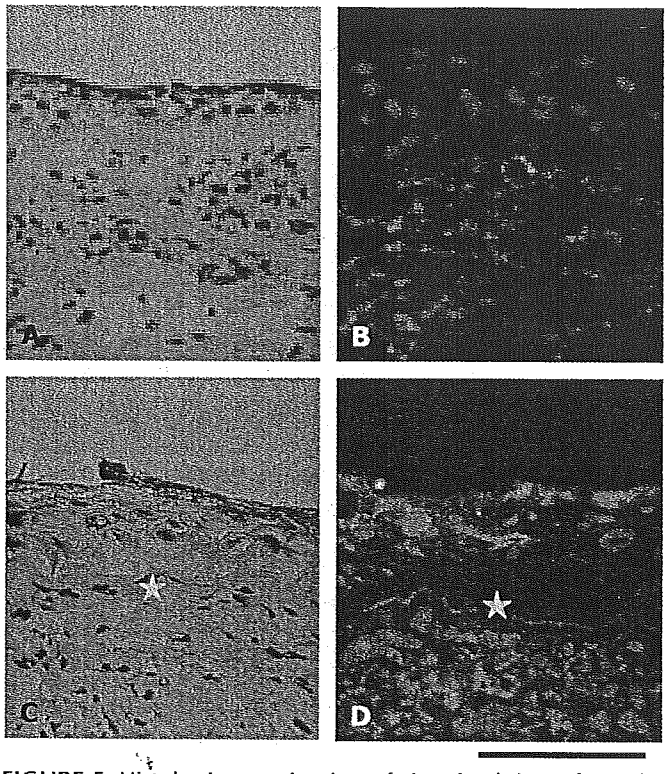

FIGURE 5. Histologic examination of cicatrized tissue from the 2 cases shown in Figure 4. Hematoxylin and eosin staining demonstrated massive inflammatory cell infiltration in the subepithelial region (A, C). Indirect immunohistochemical examination showed negative staining of IgG (B) and positive immunoreaction of IgG along the basement membrane (D). *Basement membrane. Bar = 100 μm.

tissue transplantation onto the ocular surface is not feasible because highly vascularized oral tissue is not sufficient to replace the corneal surface. However, elimination of fibroblasts and the vascular component in the cultivation process may be a key step toward generating a cornea-like epithelial sheet in culture. We have succeeded in developing a culture system capable of generating an oral epithelial sheet on AM from small biopsy samples.[16] Using a similar procedure with a cultivated corneal epithelial sheet, a transplantable oral mucosal epithelial sheet was generated after 2–3 weeks of cultivation.

Our recent clinical study has demonstrated sufficient effect and good clinical outcome of cultivated autologous oral mucosal epithelial transplantation in severe ocular surface disorders hitherto considered contraindications for allogeneic corneal transplantation.[16] Long-term viable oral mucosal epithelial cells present cornea-like nonkeratinized stratified epithelium that sufficiently compensates physical function of the corneal surface. Long-term survival of transplanted oral mucosal epithelium provides new therapeutic options for bilateral corneal stem cell deficiency using autologous nonocular cells. We applied autologous oral mucosal epithelial transplantation to bilateral severe ocular surface diseases that had a high risk of epithelial rejection and complications, especially in young patients. Figure 6 shows the clinical results of autologous oral mucosal epithelial transplantation in a patient with bilateral acute-phase severe chemical injury and chronic stage of Stevens-Johnson syndrome from our early reports.[16] Both reconstructed corneal surfaces exhibited complete survival of oral mucosal epithelial cells over the cornea, characterized by slight differences in fluorescein staining. To some extent, peripheral revascularization is commonly observed in all cases of cultivated oral mucosal epithelial transplantation as a result of different cellular response to postoperative inflammation and extracelluar matrix interaction. To prevent the use of immunosuppressive reagents and associated complications, cultivated oral mucosal epithelial transplantation is the clinically proven procedure.

OCULAR SURFACE RECONSTRUCTION WITH AUTOMATED LAMELLAR THERAPEUTIC KERATOPLASTY

Combined surgical procedures such as cataract surgery and lamellar keratoplasty are commonly required at the time of cultivated mucosal epithelial transplantation. However, these procedures are not easily compared with the single procedure for nonocular surface disease. In particular, corneal opacity interferes with transparency that is essential for intraocular handling. Thus, special surgical modifications that increase visibility and reduce refluxing illumination are necessary for safe cataract surgery. Lamellar keratoplasty and deep lamellar keratoplasty are effective procedures to remove scarred corneal opacity. Automated lamellar therapeutic keratoplasty (ALTK) is a newly introduced surgical procedure based on microkeratome-assisted keratoplasty and provides smooth and sharp stromal excision. This surgical procedure is also very useful to improve intraoperative visibility at the time of cataract surgery in patients with hazy cornea. The surgical method carried out on an eye with aniridia is shown in Figure 7. In this case, cataract surgery was impossible due to severe whole corneal opacity. However, once the cornea was excised with a microkeratome after removal of conjunctivalized tissue, clarity improved sufficiently enough for cataract surgery. Staining the anterior lens capsule with indocyanine green and surgical slit-lamp illumination are effective procedures when performing cataract surgery under poor visibility conditions. An identically sized corneal graft was created using Moria ALTK system and sutured with 10-0 nylon. Then the corneal surface was covered with cultivated corneal epithelium, resulting in improvement of visual acuity to 20/200. In addition to new cultivated epithelial transplantation, combined surgery using new instruments has resulted in improved clinical outcome and postoperative visual quality.

CONCLUSION

Since the beginning of this century, concepts and techniques of corneal surgery have widely changed following increased understanding of the biologic characteristics of ocular surface, as well as development of new devices and laboratory work. Although the ideal outcomes of each treatment vary among different diseases and ocular conditions, improved visual rehabilitation and long-term maintenance are common goals in corneal surgery. Minimal replacement of corneal parts