Amniotic Membrane Transplantation with Conjunctival Autograft for Recurrent Pterygium

Jun Shimazaki, MD, Koichi Kosaka, MD, Shigeto Shimmura, MD, Kazuo Tsubota, MD

Objective: To study the effect of amniotic membrane transplantation (AMT) combined with either limbal autograft transplantation (LAT) or conjunctival autograft transplantation (CAT) in recurrent pterygium.

Design: Retrospective, noncomparative, interventional case series.

Participants: Twenty-seven eyes of 27 patients with recurrent pterygium. The mean number of prior surgeries was 3.1 (range, 1–10). Fifteen eyes each had restriction of ocular movement and symblepharon before surgery.

Intervention: Patients were treated by AMT with either LAT (n = 15) or CAT (n = 12).

Main Outcome Measures: Recurrence of pterygium, improvement in ocular movement, and symblepharon formation.

Results: Twenty-three (85.2%) of 27 eyes showed no recurrence with a mean observation period of 67.0 weeks. Fourteen eyes (93.3%) each showed improvement in ocular movement restriction and symblepharon after AMT. In four eyes that developed recurrence, three had LAT and one had CAT combined with AMT, suggesting that there was no difference in surgical outcome between LAT and CAT.

Conclusions: AMT with CAT is a safe and effective method for recurrent pterygium, especially that associated with ocular movement restriction and symblepharon. Considering the potential adverse effects associated with limbal excision, AMT plus CAT may be preferred over AMT plus LAT. *Ophthalmology 2003;110:119–124 © 2003 by the American Academy of Ophthalmology.*

Recurrent pterygium is a challenging ocular surface disorder that is often resistant to conventional surgeries. Although various surgical approaches have been advocated, recurrence is still common, with an incidence ranging up to 55%.[1–5] Subconjunctival fibrosis is often associated with recurrent pterygium, which sometimes results in either restriction of ocular movement or adhesion between the eyelid and eye (symblepharon).

Transplantation of preserved human amniotic membrane (amniotic membrane transplantation; AMT) has recently been introduced in ocular surface reconstruction.[6] AMT has been shown to be effective for cicatricial keratoconjunctivitis, conjunctival defect, persistent epithelial defects, bullous keratopathy, corneal ulcer, and corneal perforation.[7–17] One of the remarkable features of AMT is the suppression of postsurgical fibrosis. The use of amniotic membrane in pterygium treatment has been reported.[10,12,18,19] Our group reported four cases of recurrent pterygium associated with symblepharon that were successfully treated by AMT plus limbal autograft transplantation (LAT).[10] Encouraged by this preliminary result, we conducted a large-scale study for patients with recurrent pterygium. In addition to the prevention of recurrence, the effects on fibrosis formation after AMT were studied. The efficacy of conjunctival autograft transplantation (CAT) plus AMT was also studied with the comparison to LAT plus AMT.

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Department of Ophthalmology, Tokyo Dental College, Chiba, Japan.

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Correspondence to Jun Shimazaki, MD, Department of Ophthalmology, Tokyo Dental College, 5-11-13 Sugano, Ichikawa-shi, Chiba, 272-8513, Japan. E-mail: jun@eyebank.or.jp

Materials and Methods

Subjects

Twenty-seven consecutive eyes of 27 patients with recurrent pterygium treated by AMT with either LAT or CAT between July 1995 and May 2001 in the Tokyo Dental College were enrolled. A chart review of these patients was made in this retrospective study. All patients were treated by two of the authors (JS and SS). Written informed consent was obtained from each patient after the purpose and potential risks of the procedure were explained. Four of these eyes had been reported previously.[10] Patients with a follow-up period of <6 months or pterygium secondary to injury (pseudopterygium) were not included. Patients consisted of 12 males and 15 females, with a mean age of 52.4 ± 15.2 years (range, 18–81 years). All eyes had previous surgery for pterygium, with a mean number of 3.1 times (range, 1–10). Eighteen eyes (66.7%) had previous pterygium surgery more than once. The previous surgical

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Table 1. Demographic, Clinical,

Patient No.	Age (yrs)/ Gender	Previous Treatments	Preoperative					CAT/LAT
			Size (mm)	CVA	EOM	Diplopia	Symblepharon	
1	56F	2	2	1	+	2	+	AMT
2	50F	7	3	1.2	+	3	+	LAT
3	46M	5	5	0.4	−	0	+	LAT*
4	26F	1	1.5	1.2	+	1	−	LAT
5	30F	2	4	1.2	−	0	−	LAT
6	56M	3	4	0.9	+	3	+	LAT
7	57F	1	2	0.9	−	0	−	LAT
8	57M	1	3	1.2	+	1	−	LAT
9	40F	3	5	0.6	−	0	+	LAT
10	43M	4	6	0.7	−	0	−	LAT
11	58F	1	1	1	−	0	+	LAT
12	73F	1	3	0.6	−	0	−	LAT
13	65M	3	4	0.7	+	1	+	LAT
14	49M	1	4	1.2	+	2	+	LAT
16	50F	10	4	1	+	3	+	LAT
15	58M	4	6	0.8	+	2	+	CAT
17	69F	2	7	0.4	+	3	+	CAT
18	32M	1	2	1.2	−	0	−	CAT
19	52F	1	4	0.8	−	0	−	CAT
20	68M	4	1	0.4	+	2	+	CAT
21	42M	1	3	1.2	−	0	−	CAT
22	51F	2	4	1.0	+	2	−	CAT
23	18F	2	2	1.2	−	0	−	CAT
24	50F	4	6	0.7	+	2	+	CAT
25	77M	2	3	0.2	+	1	+	CAT
26	81F	7	4	0.5	+	NA	+	CAT
27	62M	3	3	0.6	+	2	−	CAT
Mean	52.4	3.1	3.57	0.78				

AMT = amniotic membrane transplantation; CAT = conjunctival autograft transplantation; CVA = corrected visual acuity; EOM = restriction of mitomycin C; NA = not applicable.

methods included simple excision, conjunctival flap, lamellar keratoplasty, keratoepithelioplasty, and application of mitomycin C (MMC). Demographic, clinical, and surgical data are shown in Table 1.

Preparation of Human Amniotic Membrane

The use of human amniotic membrane for ocular surface diseases was approved by the institutional review board in the Tokyo Dental College. Human amniotic membrane was obtained and prepared as previously described.[7,8] Briefly, amniotic membrane with placenta was obtained at the time of elective cesarean section. Written informed consents were obtained from mothers whose serologic tests were negative for hepatitis B and C virus, human immunodeficiency virus, and human T-cell lymphotropic virus infection. Amniotic membrane with chorion was manually separated and then washed with physiologic saline containing 50 mg of dibekacin sulfate (Panimycin, Meiji Pharmaceutical Co., Tokyo, Japan). The membrane was cut into small pieces, which were immersed in sequential concentrations (0.5, 1.0, and 1.5 M) of dimethyl sulfoxide and placed into microtubes. Samples were stored at −80° C and were thawed and washed with physiologic saline immediately before use.

Surgical Methods

Most of the surgery was performed under retrobulbar anesthesia. First, fibrotic tissue was extensively dissected to expose scleral and corneal stroma. In eyes with massive fibrosis involving the medial rectus muscle, strabismus hooks were used to isolate the muscle. MMC was used during surgery in five eyes that had a history of multiple previous surgeries (four of five eyes had had surgery at least four times) associated with massive subconjunctival fibrosis. MMC was used at a concentration of 0.02% for 3 minutes, followed by irrigation with 300 ml of balanced salt solution. Amniotic membrane was then placed over the entire excised area with the epithelial side facing upward. The membrane was fixed on the sclera with 8-0 Vicryl sutures (Johnson & Johnson Co., Somerville, NJ). Amniotic membrane was placed only on the sclera, and the corneal portion was trimmed in all except two cases, in which the membrane was also secured on the cornea with use of 10-0 nylon sutures.

After AMT, either LAT (n = 15) or CAT (n = 12) was performed. Donor tissue approximately 5 × 5 mm in size was excised from the upper bulbar conjunctival area of the affected eye. In one eye, the limbal tissue was excised from the opposite eye because the affected eye had conjunctival scarring due to previous conjunctival excision. In LAT, a surgical blade (Micro-Feather Blade K-730, Feather Safety Razor Co., Tokyo, Japan) was used to excise limbal tissue as thinly as possible (approximately 1 mm wide). The conjunctival or limbal graft was placed on the amniotic membrane and secured with 10-0 nylon and 8-0 Vicryl sutures in the limbal or conjunctival area, respectively. The grafts were placed on the area where pterygia invaded the cornea, and they covered only the perilimbal portion of the amniotic membrane (Fig 1). At the end of surgery, a therapeutic soft contact lens was placed, and subconjunctival injection of antibiotics (dibekacin sulfate) and corticosteroid (betamethasone, Rinderon; Shionogi Pharmaceutical Co., Osaka, Japan) was performed.

and Surgical Data of the Patients

	Postoperative							
MMC	*CVA*	*EOM*	*Diplopia*	*Symblepharon*	*Recurrence*	*Reoperation*	Follow-up (days)
–	0.8	+	2	Slight	–		2130
–	1.2	+	1	+	–	AMT	1208
–	1	–	0	–	+	AMT + LKP + MMC	298
–	1.2	–	0	–	–		228
–	1.2	–	0	–	–		219
–	0.9	–	0	–	+		238
–	1.2	–	0	–	–		316
–	1.2	–	0	–	–		373
–	1	–	0	Slight	+	AMT + CAT + MMC	1320
–	1.2	–	0	–	–		187
–	1.2	–	0	–	–		337
–	0.7	–	0	–	–		191
–	0.7	–	0	Slight	–		571
–	1.2	–	0	–	–		276
+	1	+	1	–	–		317
+	1.2	–	1	–	–		338
–	0.3	+	0	Slight	–		225
+	1.2	–	0	–	+	AMT + CAT + MMC	784
–	0.8	–	0	–	–		649
+	0.4	–	0	Slight	–		469
–	1.2	–	0	–	–		294
–	1.2	–	1	–	–		251
–	1.2	–	0	–	–		339
–	0.5	–	1	Slight	–		190
–	0.7	–	0	–	–		180
+	0.5	–	0	Slight	–		214
–	0.7	–	0	–	–		512
	0.89						468.7

extraocular movement; *obtained from opposite eye; F = female; LAT = limbal autograft transplantation; LKP = lamellar keratoplasty; M = male; MMC =

Examination

In addition to routine ophthalmic examinations, including visual acuity, intraocular pressure measurement, and fundus examination, the following tests were performed before and after surgery. The length of pterygium invasion on the cornea and the presence or absence of symblepharon formation was recorded by using slit-lamp biomicroscopy. In cases associated with diplopia, the double-vision chart test, which examined the area of diplopia within 40° from the central fixation point, was performed. The results were recorded semiquantitatively by using the following criteria: grade 0, no area of diplopia; grade 1, diplopia within 40°, but not within 20°, of the fixation point; grade 2, diplopia present within 20°, without involvement of the central gazing point; and grade 3, diplopia involving the gazing point. Restriction of ocular movement was examined by the Hess screen chart test. Recurrence of pterygium was defined as fibrovascular tissue invasion beyond the corneal limbus.

Results

After the surgery, the surgical area epithelialized within 2 weeks, and none of the transplanted amniotic membrane sloughed off. Donor sites were promptly epithelialized with no or minimal scar formation, except in one eye, in which a visible scar formed at the donor site (case 3). No late epithelial breakdown was observed. With a mean follow-up of 67.0 weeks, 23 (85.2%) of 27 eyes showed no recurrence. Typically, the surgical area was smooth and white and showed very limited fibrosis (Figs 2 and 3). Four of the five eyes with intraoperative application of MMC did not demonstrate recurrence.

Among four eyes that had rerecurrence, fibrovascular tissue grew around the amniotic membrane in two eyes and over the graft in two other eyes. One of these four eyes (case 6) did not require further surgery because the corneal invasion was limited and stable. Three other eyes received additional surgery, two (cases 9 and 18) with repeated AMT plus conjunctival autografting with intraoperative use of MMC. The other eye (case 3) had AMT plus lamellar keratoplasty using a preserved donor cornea. Two of these three eyes showed recurrence with limited corneal invasion, and no further surgery was needed. One other eye (case 2) with persistent symblepharon received repeated AMT after excision of scar tissue 11 months after the first AMT. Although the degree of symblepharon was improved by this reoperation, slight adhesion between the upper lid and bulbus remained.

In 15 eyes that had symblepharon before surgery, complete remission was observed in 7 eyes; symblepharon partially remained but improved in 7 eyes, and only 1 eye (case 2) showed persistence of symblepharon. Changes in diplopia are summarized in Table 2. All 12 eyes that did not have ocular movement restriction before surgery remained free from diplopia after surgery. Out of 15 eyes that had ocular movement restriction, all but 1 eye (93.3%) demonstrated improvements in diplopia, with 9 eyes showing complete remission after surgery. Corrected visual acuity improved in 6 eyes, was unchanged in 19 eyes, and decreased in 2 eyes. The mean acuity changed from preoperative values of 0.78 to 0.89 after surgery.

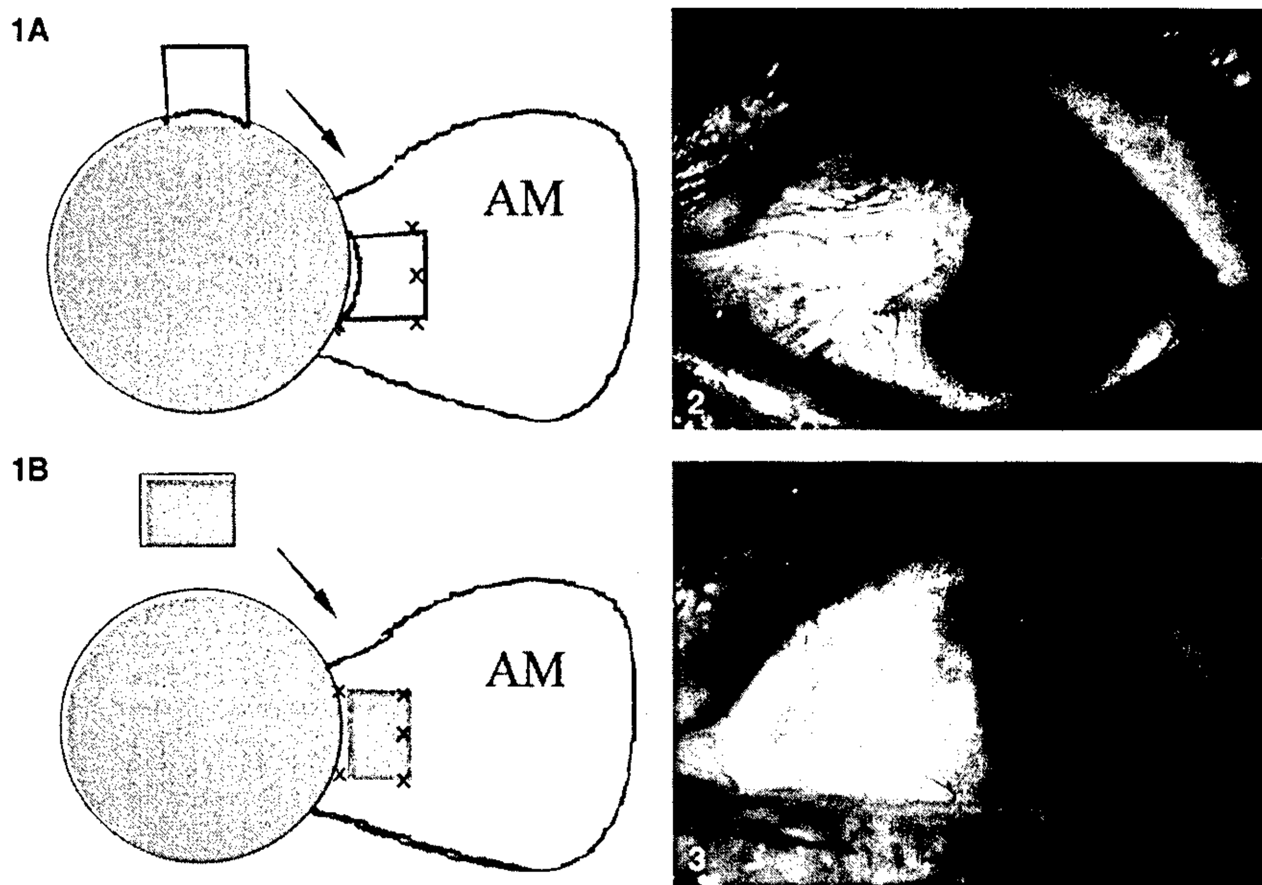

Figure 1. Diagrammatic representation of amniotic membrane transplantation (AMT) with limbal autograft transplantation (A) and AMT with conjunctival autograft transplantation (B). Note that the conjunctival or limbal graft covers only the perilimbal portion of the amniotic membrane (AM).

Figure 2. Preoperative appearance of recurrent pterygium in case 27. Note that fibrosis involves the medial rectus.

Figure 3. Postoperative appearance of the same patient as in Figure 2. Although superficial stromal opacity with vascular invasion persisted around the conjunctival graft, fibrosis was markedly suppressed.

Surgical outcomes in eyes receiving AMT plus LAT and AMT plus CAT are summarized in Table 3. Although the latter procedure produced better results in recurrence rate (20.0% vs. 8.3%), there was no statistically significant difference.

Discussion

Recurrent pterygium is characterized by hyperproliferation of subconjunctival fibroblasts, resulting in fibrosis with a more accelerated growth rate than primary pterygium. The fibrosis sometimes involves the medial rectus muscle, causing restriction in ocular movement, symblepharon formation, or both. Excision of fibrous tissue with or without conjunctival flap is often insufficient because the underlying disorder, subconjunctival fibrosis, is not treated.

In this study, the combination of AMT with either LAT or CAT successfully treated recurrent pterygium in >80% of cases. Many of the cases treated were complicated by multiple surgeries that resulted in massive fibrosis. In fact, all three eyes that required reoperation in our series had had at least three previous pterygium surgeries. Although 18 of 27 eyes had either ocular movement restriction or symblepharon before surgery, all but 1 eye demonstrated improvement (Tables 1 and 2). The results seem to indicate a benefit of AMT for recurrent pterygium, especially that complicated by either disturbance in ocular movement or symblepharon formation.

AMT has been used for a variety of ocular surface diseases since the procedure was reintroduced by Kim and Tseng[6] in 1995 after an interval of decades.[20,21] AMT has been shown to suppress fibrosis when used for cicatricial keratoconjunctivitis and pterygium.[6–8,10–12] Tseng et al[22] demonstrated that the transforming growth factor β signaling pathway in fibroblasts was strongly suppressed when in contact with the stromal side of the amniotic membrane. Because transforming growth factor βs are potent fibrogenic growth factors, suppression of this signaling pathway has an antifibrosis effect. In addition, the amniotic membrane has an anti-inflammatory effect, which may contribute to proper postoperative wound healing.[23] We also found in our series that the surgical area was quiet, with limited scarring, after AMT (Fig 3). Because amniotic membrane has virtually no limitation in terms of its size, fibrotic tissue can be extensively excised. This advantage may also contribute to favorable surgical outcome. Amniotic membrane has very

Table 2. Changes in Diplopia

Grade Before Surgery	Grade after Surgery				
	0	1	2	3	Total
0	12	0	0	0	12
1	4	0	0	0	4
2	3	3	1	0	7
3	2	2	0	0	4
Total	21	5	1	0	27

Table 3. Summary of Surgical Outcome

Procedure	n	Recurrence (%)
AMT + LAT	15	3 (20%)
AMT + CAT	12	1 (8.3%)
Total	27	4 (14.8%)

AMT = amniotic membrane transplantation; CAT = conjunctival autograft transplantation; LAT = limbal autograft transplantation.

limited immunogenicity, because it expresses human leukocyte antigens very weakly.[24,25] No immunologic rejection against amniotic membrane has been reported after AMT. We did not find any rejection episodes, nor any other adverse effects relating to the use of amniotic membrane in our series. All the membranes epithelialized within a few weeks, and no infection was recognized.

Despite the theoretical advantages, however, clinical results of AMT for pterygium have been variable. Prabhasawat et al[12] reported a recurrence rate of 37.5% (three of eight eyes) in recurrent pterygia treated by AMT. The rate was significantly higher than those treated by conjunctival autograft. The authors later reported a better result, with a recurrence rate of 9.5%, after modification of the surgical method.[19] The modified procedure included an extensive excision of pterygial tissue and subconjunctival injection of corticosteroid. The improved results suggest that suppression of fibrosis and inflammation is the key to successful treatment for recurrent pterygium. In this study, we placed the conjunctival or limbal graft on the amniotic membrane to facilitate conjunctival epithelialization. We used conjunctival or limbal grafts approximately 5 × 5 mm in size. Although the grafts were not large enough to cover the entire amniotic membrane, epithelialization seemed to be achieved promptly after surgery. We assumed that prompt epithelialization was vital for suppression of inflammation. Our favorable surgical outcome may be attributed to this surgical modification. Because we placed sutures on the conjunctival flap that anchored both the amniotic membrane and sclera, the flap may also have functioned as a mechanical barrier against fibrous tissue invasion. In the initial half of the cases, we performed LAT and later switched to CAT. Through the observation of eyes after LAT, we found that regrowth of fibrous tissues seemed to be blocked either by amniotic membrane or the conjunctival, but not the limbal, portion of the grafts. Therefore, we used CAT in the later series, expecting that the conjunctival grafts would have barrier effects similar to those of limbal grafts, although LAT has been shown to be effective for recurrent and advanced pterygium.[26] As a result, we did not find a significant difference between LAT and CAT in clinical outcome. Considering the possible adverse effects associated with excision of limbal tissue, we recommend CAT rather than LAT when combined with AMT.

An alternative approach for subconjunctival fibrosis is to use antimetabolites such as 5-fluorouracil and MMC. This method has strong antifibrosis effects and showed excellent results in pterygium treatment.[27–31] We used MMC for cases that had undergone previous multiple surgeries that were complicated by recurrence with severe subconjunctival fibrosis. We found that four of the five eyes in which we used intraoperative MMC were free from recurrence. However, we do not recommend using MMC in all cases, because we are not fully convinced that the use of antimetabolites in pterygium surgery is completely safe.[32,33] We currently consider that the use of MMC is justified only when AMT plus CAT fails. Further study is needed to clarify this point.

In summary, we found that AMT with conjunctival autografting is a safe and effective surgical method for the treatment of recurrent pterygium, especially that associated with ocular movement restriction, symblepharon, or both. Prospective, randomized clinical trials are needed to elucidate the advantages of each treatment method (AMT, CAT, and LAT) and the use of MMC.

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Collagen-Poly(*N*-Isopropylacrylamide)–Based Membranes for Corneal Stroma Scaffolds

Shigeto Shimmura, MD, Charles J. Doillon, MD, PhD, May Griffith, PhD, Masatsugu Nakamura, PhD, Edith Gagnon, BSc, Akemi Usui, PhD, Naoshi Shinozaki, PhD, and Kazuo Tsubota, MD

Abstract

Purpose:
To investigate the feasibility of using the biocompatibility of collagen-based blended biomaterials as cell-delivery systems in ocular surface reconstruction in vivo.

Methods:
Collagen-based composites that were blended with synthetic acrylamide-based polymers [poly(*N*-isopropylacrylamide), pNIPAAm] were transplanted into corneal pockets of white rabbits, with a 3-mm epithelial window. Epithelial cells were allowed to migrate onto the polymer. Transplanted eyes were examined daily for up to 30 days, after which animals were killed for histologic examination. Immunohistochemistry was performed for vimentin, α–smooth muscle actin (α-SMA), CD4, and CD8. Gold-chloride staining was performed to observe neuronal regrowth. Human amniotic membranes (AMs) and sham-operated corneas served as controls. All animals received topical antibiotics (levofloxacin) without the use of steroids or other immunosuppressive agents.

Results:
The pNIPAAm polymer allowed smooth epithelialization of the cornea, which was similar to the epithelialization observed in sham controls and AM-transplanted eyes. Histology revealed that epithelium overlying the polymer was bundled into several layers, without the orientation observed with AM and sham controls. The polymer gradually thinned and was gradually replaced by host tissue. Vimentin- and α-SMA–positive cells were found in stromal pockets up to 1 month following polymer transplantation. These cells were responsible for slight subepithelial haze near the wound edge. CD4- and CD8-positive lymphocytes were also observed in the vicinity of the polymer. Gold-chloride staining showed nerve regrowth in the wound edge after 1 month and subepithelial branches after 3 months.

Conclusion:
Collagen–pNIPAAm blended polymers may be effective as biomaterials to be used in the early stages of lamellar stromal replacement.

Key Words: collagen, cornea, isopropylacrylamide, keratoplasty, polymer

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Damage to the cornea by injury or disease can lead to loss of transparency. In cases where this transparency loss is irreversible, loss of vision or corneal blindness is a result. At present, corneal blindness is treated by transplantation of donated human corneas through penetrating keratoplasty. However, when the damage is confined to the superficial epithelial layers of the cornea, penetrating keratoplasty is unnecessary. In the past few years, following the identification of putative corneal stem cells at the limbal rim and the introduction of limbal transplants to resurface damaged corneas, ocular surface reconstruction has become increasingly popular. Where the patient's own stem cells are unavailable, donor stem cells have been used. This generally requires the use of human amniotic membranes (AMs) as delivery systems for these stem cells. Human AMs have also been used as replacements for corneal tissue. However, although corneal architecture is restored, in the long term, pigment deposits and vascularization have been reported within the deep layers of the cornea. In addition, human AMs are susceptible to infectious contamination and transmission. In

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From the Department of Ophthalmology (Drs Shimmura, Usui, Shinozaki, and Tsubota), Tokyo Dental College, Ichikawa General Hospital Cornea Center, Ichikawa, Chiba, Japan; University of Ottawa Eye Institute (Dr Griffith), University of Ottawa, Ottawa, Ontario, Canada; Santen Pharmaceutical Co. Ltd. (Dr Nakamura), Nara R&D Center, Ikoma-shi, Nara, Japan; and Laval University Medical Center (CHUL) (Drs Doillon and Gagnon), Laval University, Quebec, Canada.

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Drs Shimmura and Doillon contributed equally to this work.

A US provisional patent application has been filed.

Reprints: Shigeto Shimmura, MD, Department of Ophthalmology, Tokyo Dental College, Ichikawa General Hospital, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan (e-mail: shimmura@tdc.ac.jp).

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this study, we evaluated the feasibility of using collagen-poly(*N*-isopropylacrylamide) (pNIPAAm)-based membranes as cell-delivery systems in ocular surface reconstruction, as a replacement for corneal stroma tissue.

The pNIPAAm family of polymers is one of the few synthetic materials that support cell ingrowth and growth of polymer-encapsulated cells without any cytotoxic effects.[1,2] The pNIPAAm polymer has been proposed for use in surgical repairs because of its thermal-responsive properties in various tissue-engineered systems such as cartilage.[3] It has not been used before in ocular tissue engineering, nor has it been previously combined with collagen to produce a scaffold as described in this study.

At present, after penetrating keratoplasty with human donor corneas, there is a lack of nerve growth into the graft. This loss of innervation has been shown to contribute to the development of dry eye and/or loss of corneal sensitivity. Because of the importance of nerves in the maintenance of a healthy ocular surface, we evaluated the potential of these membranes to promote nerve regeneration and ingrowth into the new epithelium following surgery.

MATERIALS AND METHODS

Preparation of Membranes

A sterile solution of type I collagen from rat tail [3.0-3.5 mg/mL (weight/vol) in 0.02 M acetic acid] was blended with a 1% pNIPAAm solution in sterile water in a 1:1 ratio (vol/vol) at 4°C to prevent premature gelification or fibrillogenesis of the collagen during this procedure. Blended collagen-pNIPAAm was then poured into a sterile Petri dish and air-dried under sterile conditions for at least 2-3 days at room temperature to a constant weight of approximately 7% water residue. Before use, the dried membrane was completely rehydrated by soaking in a sterile buffered Hanks balanced salt solution at room temperature.

Corneal Implants

Following the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, collagen-pNIPAAm membranes were transplanted into circular pockets within the central corneal area of 24 female Japanese white rabbits (weight 3.0-3.5 kg; CLEA Japan Inc., Tokyo, Japan). A 3-mm circular central wound was created using a trephine to make an incision and then peeling off the anterior stroma and epithelium (Fig. 1). Transplanted eyes were observed by slit-lamp examination for up to 3 months, after which animals were killed for histologic examination. Corneas were evaluated for epithelial ingrowth into the wound areas over the membranes, immune and inflammatory reactions caused by the membranes, cell ingrowth from the host tissue, and regeneration of nerves in the new epithelium. Control corneas either received sham surgery (n = 6) or were implanted with human AMs (n = 20). All animals received topical antibiotics (levofloxacin) without the use of steroids or other immunosuppressive agents.

Immunohistochemistry

Rabbits were killed with an overdose injection of pentobarbital. Excised corneoscleral rims were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight and embedded in paraffin wax. After deparaffinization, sections were treated with 0.3% hydrogen peroxide in methanol at room temperature for 30 minutes to block endogenous peroxidase activity. Sections were fixed and then washed with 0.01 M phosphate buffer saline, again at pH 7.4. For immunohistochemical staining of CD4 and CD8, the specimens were embedded in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). Cryosections (5-μm slices) were fixed with cold acetone for 10 minutes.

Tissue sections were treated with anti-rabbit CD4 (KEN-4; Spring Valley Laboratories Inc., Woodbine, MD) and anti-rabbit CD8 (12.C7; Spring Valley Laboratories Inc.)

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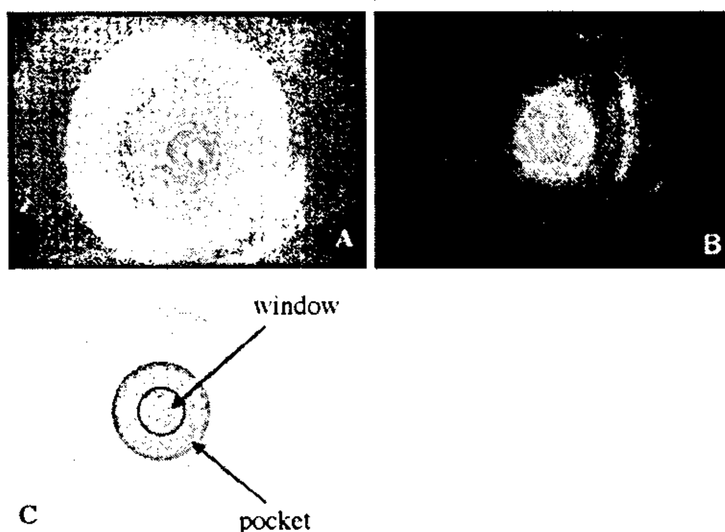

FIGURE 1. Schematic diagram and slit-lamp photograph (A) of 6.0-mm diameter polymer inserted into the stromal pocket of a Japanese white rabbit. B, The central 3.0-mm window stained with fluorescein immediately following surgery.

antibodies as well as anti-pig vimentin (V9; Laboratory Vision Corporation, Fremont, CA) and anti-α-SMA (1A4; Neo Markers, Fremont, CA). Staining was done by the avidin-biotin complex method using commercial staining kits (SAB-AP or SAB-PO; Nichirei, Tokyo, Japan). The sections for vimentin were boiled in 10 mM citrate buffer (pH 6.0) for 10–15 minutes, followed by cooling at room temperature for 20 minutes. Anti-CD4 (1:1000) and anti-CD8 (1:500) antibodies were reacted at 4°C overnight and incubated with biotinylated goat anti-mouse IgG2a and biotinylated goat anti-mouse IgG1, respectively. Sections were visualized with alkaline-phosphatase substrate using the SAB-AP kit. Reaction with antivimentin (1:200) was at room temperature for 60 minutes, and anti-α-SMA (1:200) at room temperature for 30 minutes. Sections were then reacted with biotinylated goat anti-mouse IgG (heavy chain and light chain) and biotinylated goat anti-mouse IgG2a, respectively, and developed with diaminobenzidine using the SAB-PO kit.

Gold-Chloride Stain

Rabbits were killed as described above, and eyes were enucleated by carefully removing the bulbar conjunctiva and extraocular adnexa. After washing with saline, a 5- to 6-mm incision was created near the optic nerve trunk, and the entire globe was fixed in 10% formalin (4°C) overnight. Corneoscleral buttons were made, and six 5-mm-long radial incisions were placed around the circumference of the tissue to create flat mounts. Tissues were placed in 12-well plates containing 4 mL of 0.1 M citrate buffer (pH 3.5), epithelium side facing up. After soaking for 15 minutes, buffer was replaced with 4 mL of 1% gold chloride solution, and the mixture was allowed to react for 45 minutes at room temperature. Buttons were then placed in 50-mL microtubes containing 35–40 mL acetic acid solution, agitated at 30°C for 3 hours, immersed in 70% ethanol for 4-5 hours, 95% ethanol for 2 hours, and finally in 100% ethanol for 2 hours. Corneas were then placed in xylene for 5-minute intervals 3 times and embedded in 750 centipoise dronabinol under cover slides.

RESULTS

Corneal Implants

Regrowth of the epithelium over the central corneal defects was achieved by 3 days in 21 of 24 eyes (87.5%) in the polymer

group, 16 of 20 (80.0%) eyes in the AM group, and 6 of 6 (100%) eyes in the sham-operated group. There was no statistical difference in the wound-healing rate among groups, and both polymer and AM supported a smooth epithelial interface as visualized by slit-lamp examination (Fig. 2). Hematoxylin and eosin stains of the surgical bed showed stratified epithelium in the area of the defect, but the epithelial morphology differed among the groups (Fig. 3). Eyes implanted with AM showed normal stratification, with cuboidal basal cells that led to the wings and superficial cells oriented similarly to the normal cornea. On the other hand, cells that migrated onto implanted polymers did not show firm adhesion to the polymer, and cell stratification occurred in a more disarrayed manner. The layering effect of cells within the epithelium was lost, which resulted in less-differentiated cells filling the original wound gap. In corneas where reepithelialization was delayed, different degrees of melting were observed in areas of the polymer located underneath the healing wound (Fig. 4). The polymer was relatively intact deep within the stromal pocket. Staining with anti-matrix metalloproteinase 2 did not show immunoreactivity compared with parietal cells of the fundic gland region in the rabbit stomach (data not shown).

Antibodies against vimentin and α-SMA were used to observe keratocyte activation. The pNIPAAm-collagen polymer showed more immunoreactivity than AM, especially in the stroma located between the polymer or AM and epithelium (Figs. 5 and 6). There was less staining with both antibodies in the stroma underlying the surgical bed. The cells observed in the stroma were predominantly of keratocyte origin because immunohistochemistry against CD4 and CD8 lymphocyte markers was predominantly negative (Figs. 7 and 8).

Regeneration of nerve fibers was also observed in the rabbit models. In animals killed after 28 days, regenerating nerves were observed along the marginal edges of both AM and pNIPAAm polymers (Fig. 9). In one eye with the pNIPAAm polymer implanted for over 3 months, characteristic parallel nerve fibers were found that extended toward the epithelium in the basal layers.

DISCUSSION

Many attempts have been made to create artificial corneas or keratoprostheses to

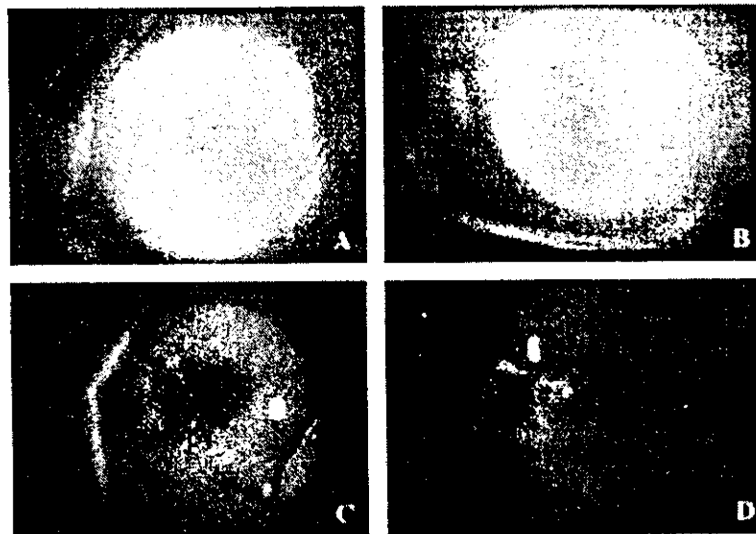

FIGURE 2. Slit-lamp micrograph of eyes implanted with AM (A,B) and polymer (C,D) at day 3. Both AM (80.0%) and polymer (87.5%) allowed prompt epithelialization by the third day.

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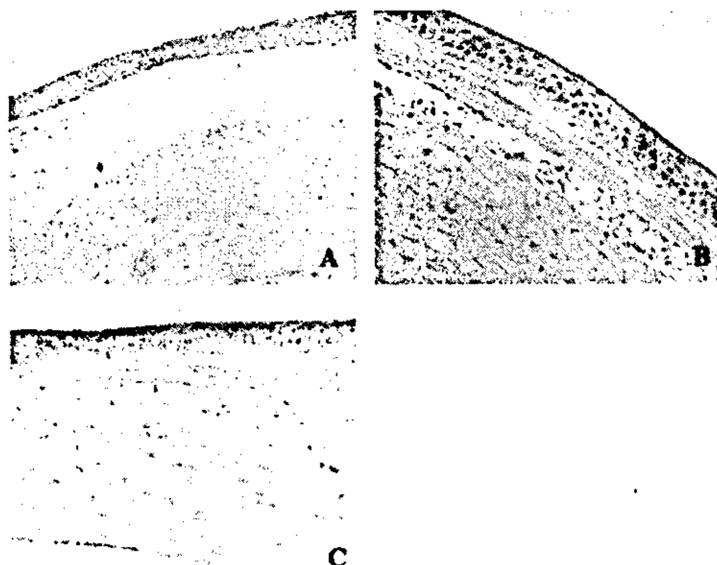

FIGURE 3. Stratified epithelium over polymer (A), AM (B), and sham surgery (C) at 28 days. The attachment of epithelium with the polymer is loose, and epithelial cell layers do not show the differentiation observed with AM and sham.

replace donor cornea grafts. They have often failed because of an absence of healing and permanent coaptation between the periphery of the synthetic device and the residual rim of host cornea. As a result, tissue necrosis, leakage of aqueous humor, epithelial downgrowth, and intraocular infection frequently occurred.

Numerous keratoprostheses have been developed using a variety of polymer materials such as poly(2-hydroxyethyl methacrylate) (pHEMA), polymethyl methacrylate, polyvinyl alcohol, or poly(ethyl vinyl alcohol), in some instances mixed with collagen or hyaluronic acid.[4-13] Most of the keratoprostheses in development consist of a central transparent optical element surrounded by a porous opaque material as a peripheral rim that allows penetration and proliferation of stromal keratocytes and the subsequent synthesis of collagen within the material. The peripheral rim has been made of different polymers such as polybutylene-polypropylene and expanded poly(tetrafluoroethylene). These devices have been more or less successful (eg, they remained in place for 6 months). However, the skirts of most of them have low tensile strength, which leads to extrusion of the keratoprosthesis, suturability remains a major problem, and there is often induction of an inflammatory reaction. One keratoprosthesis made of a transparent pHEMA core appears promising, and clinical trials are in progress.[6]

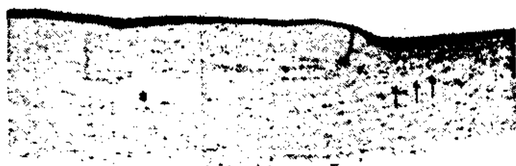

FIGURE 4. Histology section of an eye transplanted with the polymer with delayed wound healing. Although the polymer within the stromal pocket (*) was intact, most of the polymer beneath the epithelium dissolved by day 28.

The pNIPAAm family of polymers is one of the few synthetic materials that support cell ingrowth and growth of polymer-encapsulated cells.[14,15] Among preexisting patents, pNIPAAm and its derivatives have not been used in any cornea or ocular device. A thermally responsive polymer, pNIPAAm has been extensively studied in vitro for cell-culture use that demonstrated its noncytotox-

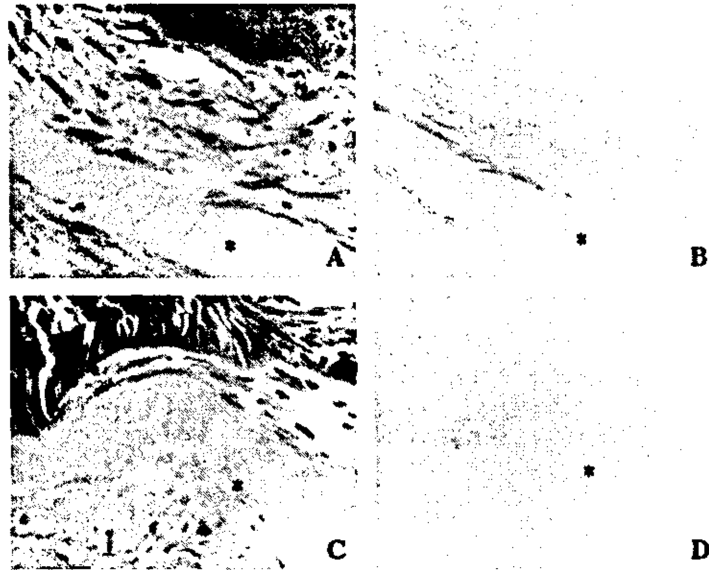

FIGURE 5. Immunohistochemistry of vimentin after 28 days. The polymer sample (A,B) shows stronger staining within the stroma compared with AM (C,D).

icity.[1,2] Collagen has been combined with pNIPAAm as a cell-culture substrate to form spheroids. They produce only a thin (2-μm) layer of collagen-pNIPAAm. Moreover, US patent no. 6,030,634[16] describes the combination of pNIPAAm with gelatin (denaturated collagen), which results in a polymer gel that has the properties of an interpenetrating polymer network structure with a shrinking temperature at 35°C; the gel has surgical application for the repair of damaged tissue but not replacement or substitution. The inventors also claim stronger gels and an improved shrinking rate for a drug delivery system using such a network structure. However, the phase transition of this pNIPAAm polymer,

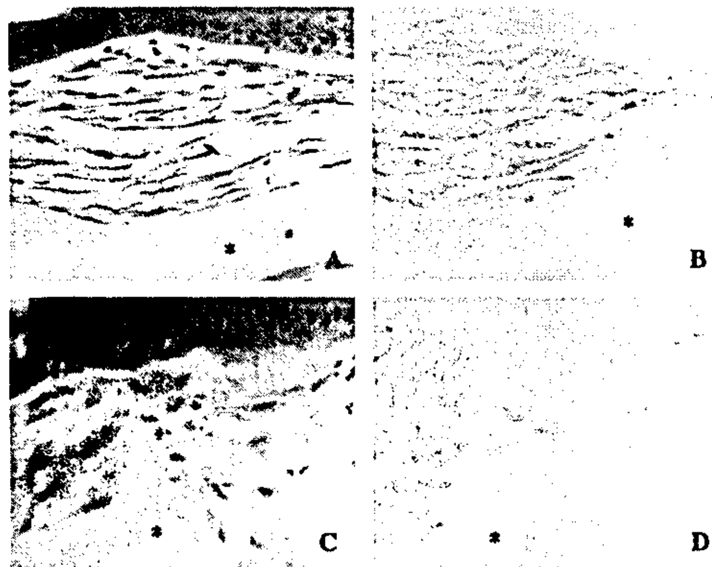

FIGURE 6. Immunohistochemistry of α-SMA after 28 days. Similarly to vimentin, the polymer (A,B) showed stronger staining than did AM (C,D). The staining was strongest in the fibroblast-like cells located between the polymer and epithelium.

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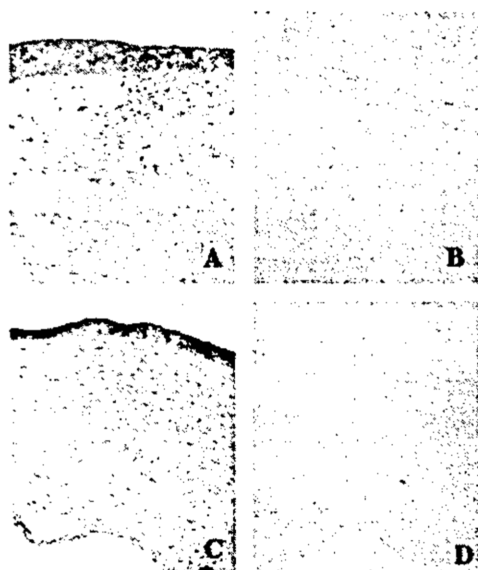

FIGURE 7. Immunohistochemistry against CD4 in polymer (A,B) and AM (C,D).

which occurs at >32°C, compromises optical clarity at body temperature. Furthermore, activated pNIPAAm has also been conjugated to protein A, various enzymes, biotin, phospholipids, Arg-Gly-Asp tripeptide sequences, and other interactive molecules such as monoclonal antibodies, as described in US patent no. 4,780,409.[17]

There are many different types of polymers and copolymers based on *N*-isopropylacrylamide (NIPAAm). The pNIPAAm homopolymer is a thermally sensitive polymer that precipitates out of water at or above 32°C, which is its lower critical solution temperature (LCST) or cloud point.[18] Because of its opacity at 37°C, the homopolymer alone is of no use as a cornea replacement. When NIPAAm monomer is copolymerized with more hydrophilic comonomers such as acrylamide or acrylic acid, the LCST is raised. The opposite occurs when it is copolymerized with more hydrophobic comonomers, such as *N-trans*-butyl acrylamide.[18]

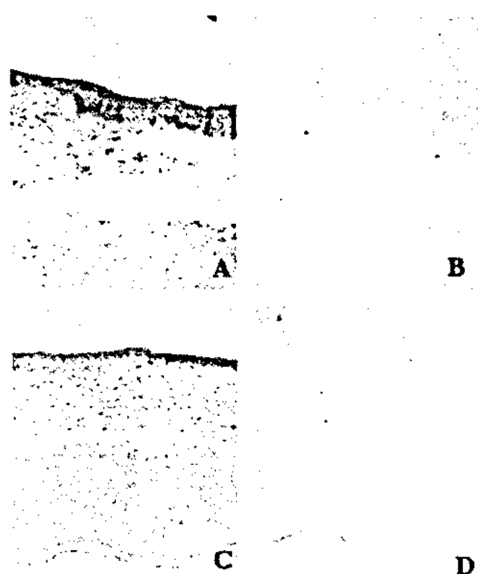

FIGURE 8. Immunohistochemistry against CD8 in polymer (A,B) and AM (C,D).

We have attempted to produce a membrane for cornea implantation by blending pNIPAAM and type I collagen. The pNIPAAm can also be modified to confer a variety of biophysical and biologic properties to the composite materials. Type I collagen may be replaced or combined with gelatin (a denaturated collagen) or peptides to enhance healing properties. The blended polymers produce a homogeneous hydrogel that becomes a transparent film or membrane on drying. The membrane or film is insoluble in physiologic liquids, transparent, and has strength with easy handling and suturability. This makes a sterile cornea-stroma equivalent that supports adhesion and migration of epithelial and endothelial cells, and their progenitors, as well as their differentiation into the desired cell. Insertion of the biopolymer as an inlay, implant, or membrane may assist wound healing in one or more layers of the human cornea.

The animal data from our study show that the pNIPAAm-collagen blend allows for prompt epithelialization of the surface. Histology revealed that epithelial differentiation overlying the polymer was abnormal. However, this can be modified by surface modification of the polymer with basement membrane components. The polymer also allows for regeneration of corneal nerves, which are crucial in maintenance of an intact ocular surface following surgery. Although the behavior of stromal keratocytes as well as endothelium in vivo still needs to be evaluated, the use of pNIPAAm-collagen blend polymers or

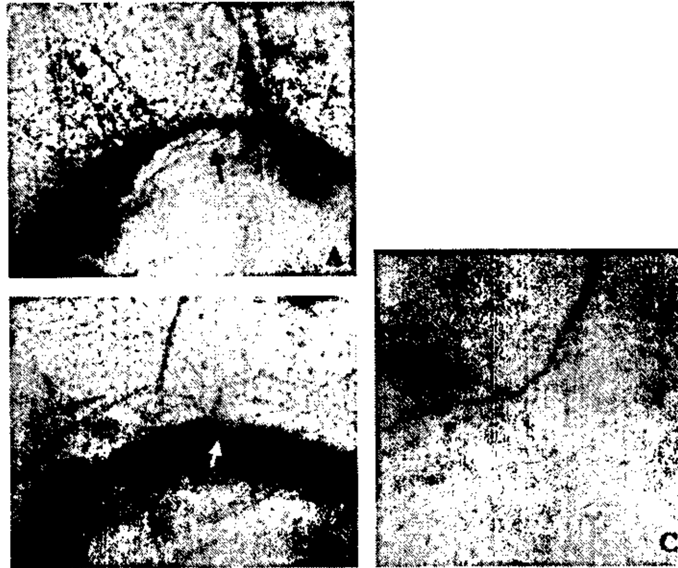

FIGURE 9. Gold-chloride stains for nerve fibers (arrows) in polymer (A) and AM (B) at day 28. After 90 days (C), parallel fibers (arrow) were observed that extended toward the epithelium in this eye implanted with the polymer.

a modification of this combination as a surgical material appears to be feasible.

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The Successful Culture and Autologous Transplantation of Rabbit Oral Mucosal Epithelial Cells on Amniotic Membrane

Takahiro Nakamura,[1] Ken-Ichi Endo,[1] Leanne J. Cooper,[2] Nigel J. Fullwood,[2] Noriko Tanifuji,[1] Masakatsu Tsuzuki,[1] Noriko Koizumi,[1] Tsutomu Inatomi,[1] Yoichiro Sano,[1] and Shigeru Kinoshita[1]

Purpose. To determine the feasibility of using human amniotic membrane (AM) as a substrate for culturing oral epithelial cells and to investigate the possibility of using autologous cultivated oral epithelial cells in ocular surface reconstruction.

Methods. An ocular surface injury was created in one eye of each of eight adult albino rabbits by a lamellar keratectomy, and a conjunctival excision was performed, including and extending 5 mm outside the limbus. Oral mucosal biopsy specimens were obtained from these eight adult albino rabbits and cultivated for 3 weeks on a denuded AM carrier. The cultivated epithelium was examined by electron microscopy (EM) and immunohistochemically labeled for several keratins. At 3 to 4 weeks after the ocular surface injury, the conjunctivalized corneal surfaces of the eight rabbits were surgically reconstructed by transplanting the autologous cultivated oral epithelial cells on the AM carrier.

Results. The cultivated oral epithelial sheet had four to five layers of stratified, well-differentiated cells. EM revealed that the epithelial cells were very similar in appearance to those of normal corneal epithelium, had numerous desmosomal junctions, and were attached to a basement membrane with hemidesmosomes. Immunohistochemistry confirmed the presence of the keratin pair 4 and 13 and keratin-3 in the cultivated oral epithelial cells. Corneas that were grafted with the cultivated oral epithelial cells on an AM carrier were clear and were all epithelialized 10 days after surgery.

Conclusions. Cultures of oral epithelial cells can be generated to confluence on AM expanded ex vivo from biopsy-derived oral mucosal tissue. Autologous transplantation was performed with these cultivated oral epithelial cells onto the ocular surfaces of keratectomized rabbit eyes. Autologous transplantation of cultivated oral epithelium is a feasible method for ocular surface reconstruction. The long-term outcome of such transplantation is not yet clear, and its feasibility in clinical use should be evaluated further. (*Invest Ophthalmol Vis Sci.* 2003;44:106–116) DOI:10.1167/iovs.02-0195

The normal ocular surface is covered with highly specialized corneal and conjunctival epithelia, which are formed by two phenotypically different types of epithelial cells.[1-5] The conjunctival epithelium is well vascularized and consists of loosely organized cell layers populated by mucin-secreting goblet cells. The cornea is covered by nonkeratinized, stratified epithelium that is responsible for maintaining ocular surface integrity and is essential for vision. Corneal epithelial cells are derived from stem cells located in the limbal epithelium.[6,7] In severe ocular surface diseases, such as Stevens-Johnson syndrome (SJS) and ocular cicatricial pemphigoid (OCP), in which limbal epithelial cells are destroyed, the neighboring conjunctival epithelial cells invariably cover the corneal surface resulting in chronic inflammation, stromal scarring, and neovascularization, which severely affects visual acuity.[2,8,9]

Many attempts have been made to establish a surgical treatment for severe ocular surface diseases.[10-14] In such diseases, because the prognosis after penetrating keratoplasty is poor, the alternative surgical treatment of corneal epithelial transplantation (limbal transplantation or keratoepithelioplasty), in conjunction with amniotic membrane (AM) transplantation, has been developed to improve the outcome of ocular surface reconstruction.[15-18] The most recently developed treatment for these diseases involves the transplantation of cultivated corneal epithelial stem cell for reconstructing the ocular surface after damage caused by corneal epithelial stem cell deficiency.[19-21] Our group developed a corneal limbal epithelial culture system, using AM as a carrier, in an animal model.[22,23] We have since adopted this system for clinical use in severe ocular surface disorders and have successfully achieved ocular surface reconstruction.[24,25] However, despite the success of these surgical procedures, several problems remain. First, transplantation of corneal epithelial cells from donors (allografts) requires sufficient donor material; second, it carries a risk of rejection. Consequently, after surgery, an intensive, long-term course of immunosuppressants must be prescribed to prevent postoperative inflammation and allograft rejection, markedly reducing the quality of life of these patients. In some cases damaged corneas have sometimes been successfully reconstructed by transplanting autologous limbal epithelial cells (autografts).[19,20] However, this is not possible in severe ocular surface diseases, such as SJS and OCP, because they are usually bilateral, and therefore autologous corneal epithelial transplantation is not an option in these cases.

This article describes an attempt to overcome the problems of allogeneic transplantation by using oral epithelial cells as a substitute for corneal epithelial cells. We cultured oral epithelial cells on AM expanded ex vivo from biopsy-derived oral mucosal tissues of eight rabbits. We then transplanted the

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From the [1]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; and the [2]Institute of Environmental and Natural Sciences, Lancaster University, Lancaster, United Kingdom.

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Corresponding author: Takahiro Nakamura, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-0841, Japan; tnakamur@ophth.kpu-m.ac.jp.

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autologous cultivated oral epithelium onto the ocular surfaces of keratectomized rabbits and evaluated the survival of the tissue. This study is a first step toward assessing the use of autologous transplantation of mucosal epithelial cells of nonocular surface origin.

Materials and Methods

Preparation of AM

With proper informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects and on approval by the Institutional Review Board of the Kyoto Prefectural University of Medicine, human AMs were obtained at the time of Cesarean section. Under sterile conditions, the membranes were washed with sterile phosphate-buffered saline (PBS) containing antibiotics (5 mL of 0.5% levofloxacin) and stored at −80°C in Dulbecco's modified Eagle's medium (GibcoBRL, Rockville, MD) and glycerol (Wako Pure Chemical Industries, Osaka, Japan) at the ratio of 1:1 (vol/vol). Immediately before use, the AM was thawed, washed three times with sterile PBS containing antibiotics, and cut into pieces approximately 4 cm × 4 cm. For the oral epithelial cultures, membranes were then deprived of their amniotic epithelial cells by incubation with 0.02% EDTA (Nacalai Tesqu Co., Kyoto, Japan) at 37°C for 2 hours to loosen cellular adhesion, followed by gentle scraping with a cell scraper (Nunc International, Naperville, IL).

Primary Cultures of Oral Epithelial Cells

We cultured the rabbit oral epithelial cells by using a previously reported culture system for corneal epithelial stem cells, with several modifications. Oral epithelial cells were cocultured with mitomycin C (MMC)-inactivated 3T3 fibroblasts. Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL MMC for 2 hours at 37°C under 5% CO_2 to inactivate their proliferative activity. They were then rinsed with PBS to remove MMC, trypsinized, and plated onto plastic dishes at a density of 2×10^4 cells/cm^2. Denuded AMs were spread, epithelial basement membrane side up, on the bottom of culture plate inserts (Corning, Inc., Corning, NY), and these inserts were placed in dishes containing treated 3T3 fibroblasts. We also used an air-lifting technique to promote oral epithelial differentiation and the epithelial barrier function.[26]

Oral mucosal biopsy specimens, each 4 to 6 mm^2 in size, were taken from eight adult albino rabbits (2-2.5kg) with anesthesia induced by intramuscular injection of xylazine hydrochloride (5 mg/mL) and ketamine hydrochloride (50 mg/mL). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the experimental procedure approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine. Submucosal connective tissues were removed with scissors to the extent possible. The resultant samples were cut into small explants that were immersed three times in PBS solution containing antibiotics: 50 IU/mL penicillin-streptomycin and 5 μg/mL amphotericin B for 10 minutes at room temperature. These explants were then incubated at 37°C for 1 hour with 1.2 IU dispase, as previously described,[27] and treated with 0.25% trypsin-EDTA solution for 30 minutes at room temperature to separate the cells. Enzyme activity was stopped by washing with culture medium comprising DMEM and Ham's F12 (1:1 mixture) with 10% fetal bovine serum (FBS), insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human recombinant epidermal growth factor (10 ng/mL), and penicillin-streptomycin (50 IU/mL). The cell suspension was filtered through a cell-dissociation sieve (Sigma, St. Louis, MO) to remove unsatisfactory segments, yielding a suspension of purified oral mucosal cells. The suspension was centrifuged twice for 5 minutes at 1000 rpm, and the resultant cell pellet was resuspended in culture medium. The oral epithelial cells (1×10^5 cells/mL) were then seeded onto denuded AM spread on the bottom of culture inserts and cocultured with MMC-inactivated 3T3 fibroblasts. The culture was submerged in medium for 2 weeks and then exposed to air by lowering the medium level (air-lifting) for 1 week. Cultures were incubated at 37°C in a 5% CO_2-95% air incubator for up to 21 days, and the medium was changed every day.

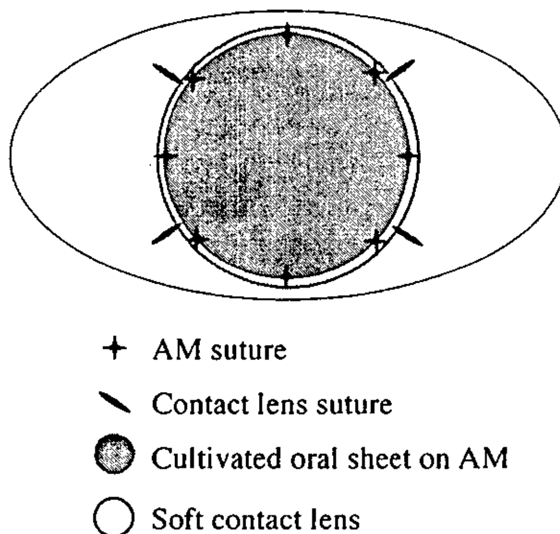

FIGURE 1. The precise placement of the AM, the sutures, and the soft contact lens is shown. The cultivated oral epithelial sheet was sutured at the corneal-limbal interface with 10-0 nylon sutures (eight sutures were always used). The soft contact lens was sutured to the bare sclera with 10-0 nylon sutures (four were always used). These sutures were not secured to the cultivated oral epithelial sheet.

Ocular-Surface Injury

To simulate the condition found in the stem cell deficiencies,[22] an ocular-surface injury was created in one eye of each of the eight adult albino rabbits by excising all the conjunctival tissue within 5 mm of the limbus and performing a superficial keratectomy of the entire corneal surface, including the limbal epithelial cells. Antibiotic eye drops (0.5% levofloxacin) and intramuscular gentamicin (1 mg/kg) were administered after surgery.

Autologous Oral Epithelial Transplantation

At 3 to 4 weeks after the ocular surface injury, the conjunctivalized ocular surfaces of the eight rabbits were surgically reconstructed by transplanting autologous oral epithelial cells cultivated on AM. In all cases, the damaged corneal surface, including the 5-mm zone of adjacent conjunctival tissue, was carefully excised under anesthesia. All animals in which oral epithelial cells had been placed in culture 3 to 4 weeks earlier received autologous cultivated oral epithelial cells on AM. All received the oral epithelial sheet as a 12-mm diameter disc of AM. The sheets were sutured to the keratectomized corneal surface with 10-0 nylon sutures and covered with a soft contact lens (58% water content) held in place by four peripheral anchoring sutures (Fig. 1). After surgery, topical antibiotics (0.5% levofloxacin) and steroids (0.1% betamethasone) were applied three times daily. For our experimental controls, four eyes received no transplant, four eyes received acellular AM transplants onto keratectomized corneas, and four eyes received the oral tissue transplant directly onto the keratectomized corneal-limbal zone.

Immunohistochemistry

Immunohistochemical studies of several keratins in the oral epithelial sheet were performed using our previously described method.[28,29] Normal rabbit cornea, conjunctiva, and oral samples were also examined for purposes of comparison. Briefly, cryostat sections (7 μm thick) were placed on gelatin-coated slides, air dried, and rehydrated in

TABLE 1. Primary Antibodies and Source

Antibodies	Category	Dilution	Source
Cytokeratin-1	Mouse monoclonal	×20	YLEM srl
Cytokeratin-10	Mouse monoclonal	×100	Biomeda Corp., Foster City, CA
Cytokeratin-3	Mouse monoclonal	×50	Progen Biotechnik GMBH, Heidelberg, Germany
Cytokeratin-12	Rabbit polyclonal	×200	Provided by Kurpakus M, et al.[60]
Cytokeratin-4	Mouse monoclonal	×10	ICN Pharmaceuticals, Inc., Costa Mesa, CA
Cytokeratin-13	Mouse monoclonal	×1	American Research Products, Inc., Kensington, MD

PBS at room temperature for 15 minutes. To block nonspecific binding, the tissues were incubated with 1% bovine serum albumin (BSA) at room temperature for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with the primary antibody (Table 1), and then washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. Control incubations consisted of incubation with the appropriate normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody and also the omission of the primary antibody for each specimen. After staining with the primary antibody, the sections were then incubated at room temperature for 1 hour with appropriate secondary antibodies, fluorescein (FITC)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and fluorescein (FITC)-conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, CA). After several washings with PBS, the sections were coverslipped using antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories) and examined by confocal microscopy (Fluoview; Olympus, Tokyo, Japan).

Electron Microscopy

Rabbit oral mucosal epithelial cells cultured on denuded AM were examined by scanning electron (SEM) and transmission electron microscopy (TEM). Normal rabbit cornea, conjunctiva, and oral samples were also examined for comparison. Specimens were fixed in 2.5% glutaraldehyde in 0.1 M PBS, washed three times for 15 minutes in PBS, and postfixed for 2 hours in 2% aqueous osmium tetroxide. They were 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., Aldermastron, UK) for 10 minutes and allowed to air dry. When dry, specimens were mounted on aluminum stubs and sputter coated with gold before examination in a digital scanning electron microscope (JSM 5600; JEOL, London, UK). For TEM, the specimens were embedded in epoxy resin (Agar 100; Agar Scientific, Ltd., Stansted, UK). Ultrathin (70 nm) sections were collected on copper grids and stained for 1 hour with uranyl acetate and 1% phosphotungstic acid and then for 20 minutes with Reynolds' lead citrate before examination by transmission electron microscope (JEM 1010; JEOL).

RESULTS

Cultivation of Oral Epithelial Cells

Epithelial cells from the oral mucosa began to form colonies on the denuded AM within 3 days. After 10 days in culture, a confluent primary culture of oral epithelial cells had been established that covered the whole AM (Fig. 2A). At 3 weeks, the cultivated oral epithelial cells showed four to five layers of stratification, were well differentiated (Fig. 2B), and appeared very similar to both normal corneal epithelium (Fig. 2C) and the corneal epithelium cultivated using our established technique (Fig. 2D).[22-27]

Immunohistochemistry of Keratins

The patterns of expression of the keratins in the cultivated oral epithelium were investigated with immunohistochemistry. Negative control sections, incubated with normal mouse and rabbit IgG, and omission of the primary antibody exhibited no discernible specific immunoreactivity over the entire region, except that there was slight nonspecific immunoreactivity for keratin-12 in the subepithelial regions of the cornea, conjunctiva, and oral mucosa. However, there was no nonspecific immunoreactivity in the epithelial regions. The immunoreactivity observed in each specimen was compared with the control. Immunohistochemistry showed the presence of keratins (4/13 and 3) in the cultivated oral epithelial cells (Figs. 3A3-3A5). The keratin-4 and -13 pair were expressed in the superficial and intermediate layers, with no discernible immunostaining in the basal cell layers. In contrast, keratin-1, keratin-10, and cornea-specific keratin-12 were not expressed in any layers of the oral epithelial sheets (Figs. 3A1, 3A2, 3A6). Cornea-specific keratin-3 was expressed in all epithelial layers (Fig. 3A5).

Immunohistochemical examination of the rabbit normal corneal, conjunctival, and oral epithelial cells showed that keratin-1 and -10 were not expressed in any layers of the corneal (Figs. 3B1, 3B2), conjunctival (Figs. 3C1, 3C2), and oral epithelial cells (Figs. 3D1, 3D2). Keratin-4 was expressed in the superficial layer (Figs. 3B3), and keratin-3 and -12 were expressed in all epithelial layers of the corneal epithelium (Figs. 3B5, 3B6). Keratin-4 and -13 were expressed in the superficial and intermediate layers of the conjunctiva (Figs. 3C3, 3C4). These keratins were expressed in all epithelial layers of oral epithelium (Figs. 3D3, 3D4), and, finally, cornea-specific keratin-3 was expressed in the oral epithelial cells (Figs. 3D5). No keratin-12 immunoreactivity was found in conjunctival (Figs. 3C6) and oral epithelium (Figs. 3D6).

Ultrastructural Features of the Epithelium

SEM examination of the cultivated oral epithelial cells revealed a continuous layer of flat, squamous, polygonal epithelial cells (Fig. 4A). These cells appeared healthy and well formed with distinct cell boundaries and ranged from 12 to 90 μm in size, the average exposed cell surface diameter was 28 ± 10.29 μm (SD). Some of the oral epithelial cells appeared to be in the process of desquamating (Fig. 4B). They were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries (Fig. 4C). The apical surface of the cells was covered with microvilli (Fig. 4D).

TEM examination of the oral epithelial culture sheet showed that the cells produced five to six layers of well-stratified epithelium (Fig. 5A), appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells (Figs. 5A, 5B). The epithelial cells in the basal cell layers were columnar (Fig. 5C), and recently divided cells provided evidence of rapid proliferation in the basal cell layers of the epithelial sheet (Fig. 5D). The basal epithelial cells adhered well to the AM substrate with hemidesmosome attachments and produced basement membrane material (Fig. 6A). In all cell layers, the epithelial cells were comparatively closely attached to neighboring cells by numerous desmosomal junctions (Fig. 6B), and in the superficial cell layer what appeared to be tight junctions were

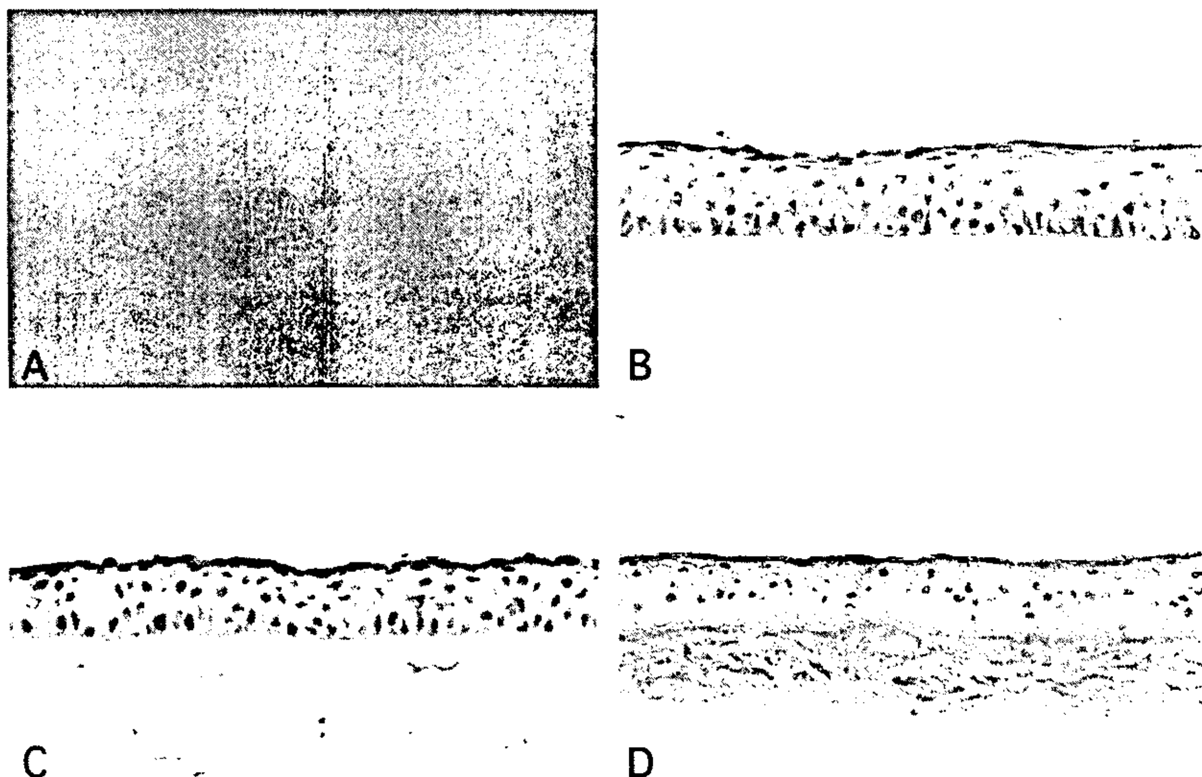

FIGURE 2. A confluent primary culture of oral epithelial cells taken from the oral tissue after 10 days in culture (A). Light micrographs showing cross-sections of the cultivated oral epithelial cells on AM (B), normal corneal epithelial cells (C), and cultivated corneal epithelial cells on AM (D), stained with hematoxylin and eosin. The cultivated oral epithelial sheet had four to five layers of stratified, well-differentiated cells and appeared very similar to both normal and cultivated corneal epithelium. Original magnification: (A) ×100, (B, C, D) ×400.

evident between neighboring cells (Fig. 6C). The apical surface of the most superficial cells was covered with a glycocalyx-like material (Fig. 6D).

SEM examination of the normal corneal, conjunctival, and oral cells showed that the surface morphology of the corneal epithelial cells was different from that of the other cell types. The corneal epithelial cells revealed a continuous layer of flat squamous polygonal epithelial cells with an average size of 28.4 ± 10.0 μm with distinct surface microvilli (Fig. 7A). The conjunctiva consisted of smaller polygonal epithelial cells with an average size of 12.3 ± 2.3 μm mixed with numerous goblet cells (Fig. 7C). The oral mucosal samples (Fig. 7E) cells had an average size of 27.5 ± 8.4 μm, and their apical surfaces had long parallel ridge-like structures quite different from the microvilli on the corneal epithelial cells.

TEM examination of the normal corneal, conjunctival, and oral cells showed that the corneal epithelial cells could be easily distinguished from the other cell types by their ultrastructure. The corneal epithelial cells consisted of five to six layers of well-stratified epithelium (Fig. 7B), appeared healthy, and were differentiated into basal columnar cells, suprabasal cuboid wing cells, and flat squamous superficial cells. The rabbit conjunctiva could be easily identified by the presence of numerous goblet cells (Fig. 7D). The oral samples (Fig. 7F) had many more cell layers than were present in the normal corneal epithelial cell layer.

Autologous Transplanted Epithelium

At 3 to 4 weeks after the ocular surface injury, conjunctival epithelium completely covered the damaged corneal surface in all eight rabbits with considerable neovascularization and subconjunctival inflammation evident (Figs. 8A1-8D1). The extent of injury was similar in all animals. After the removal of conjunctivalized tissue, we reconstructed the ocular surface with a cultivated oral epithelial sheet on AM (day 0). No signs of infection, bleeding, or sheet detachment were observed. In the early stages (day 2) after transplantation, the eight eyes that had received 12-mm diameter discs of AM containing autologous cultivated oral epithelial cells all possessed an epithelialized area (Figs. 8A2-8D2). Most of the area covered with cultivated oral epithelial cells was not stained by fluorescein and was separated from the outer rim of healing conjunctiva by an annular epithelial defect, which stained with fluorescein. At 10 days after transplantation, the area covered by the epithelium had expanded outward and was connected with healing conjunctival epithelium in some areas (Figs. 8A3-8D3). Moreover, the corneal surfaces of all eyes were clear and smooth, and the entire corneal surfaces were completely covered with transplanted autologous oral epithelium (Figs. 8A4-8D4). The control animals that had received no transplant (Fig. 8E) or had received acellular AM (Fig. 8F) did not show any evidence of epithelialization at day 10 (Figs. 8E3, 8F3). The control animals that had undergone direct transplantation of oral tissue showed considerable neovascularization and inflammation, resulting in the failure of ocular surface reconstruction (Fig. 8G).

Histologic examination of transplanted sheets at 10 days after surgery revealed that the sheets adhered well to the host corneal stroma with no evidence of subepithelial cell infiltration or stromal edema. Superficial cells of the transplanted sheets had nuclei, indicating that they were indeed nonkeratinized mucosal epithelial cells (Fig. 9).

 IOVS, January 2003, Vol. 44, No. 1

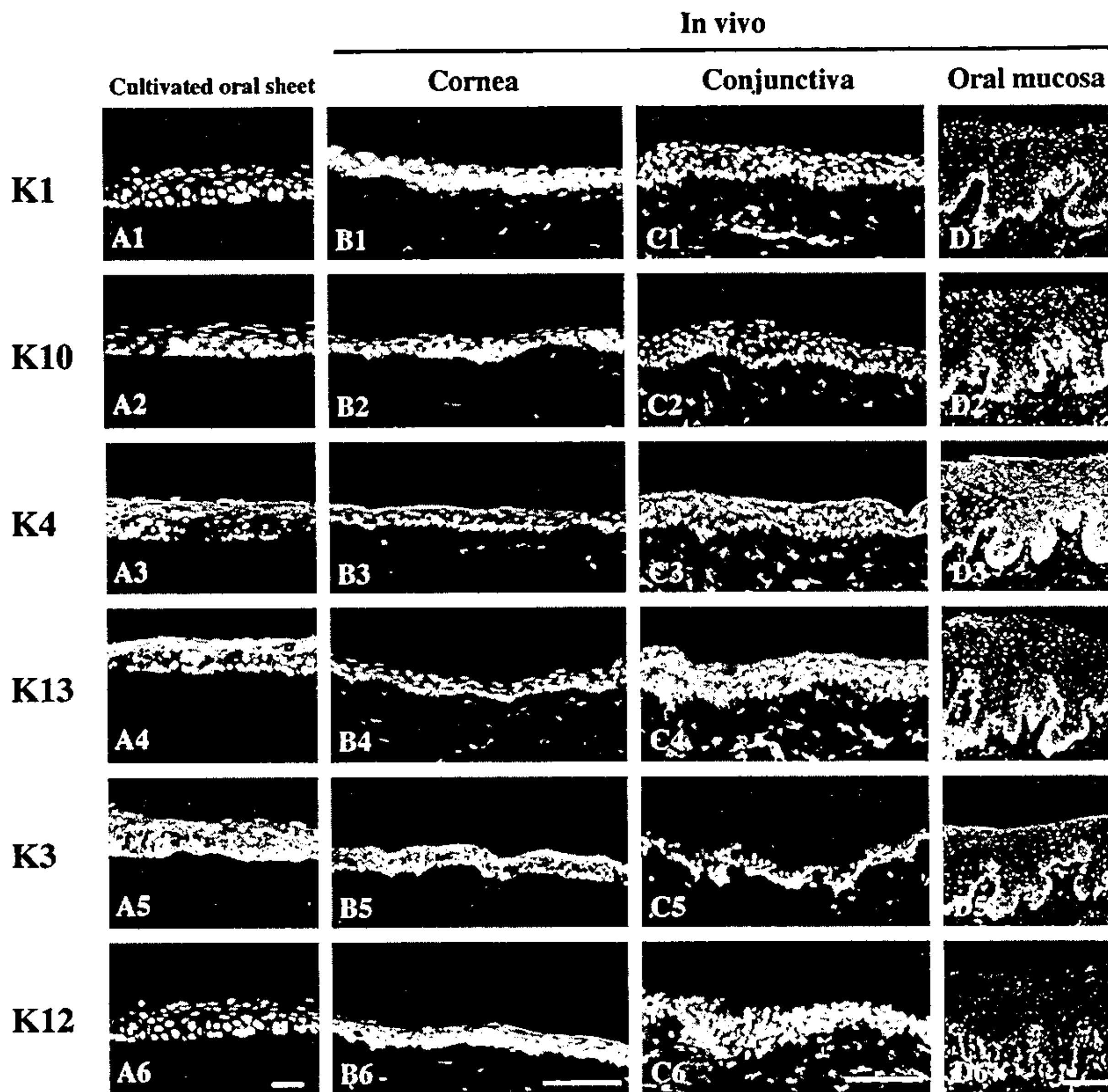

FIGURE 3. Representative immunohistochemical staining of keratins-1 (A1-D1), -10 (A2-D2), -4 (A3-D3), -13 (A4-D4), -3 (A5-D5), and -12 (A6-D6) in cultivated oral epithelial cells on AM and on normal corneal, conjunctival, and oral mucosa. Keratin-1 and -10 were not expressed in any layers of the cultivated oral epithelial cells (A1, A2) or in the normal corneal (B1, B2), conjunctival (C1, C2), or oral epithelial cells (D1, D2). In contrast, keratin-4 and -13 were expressed in the superficial and intermediate layers of the cultivated oral epithelial cells (A3, A4) and conjunctival epithelial cells (C3, C4). These keratins were expressed in all epithelial layers of oral mucosa (D3, D4), and only keratin-4 was expressed in the superficial layer of the corneal epithelial cells (B3). Keratin-3 was expressed in all epithelial layers of the cultivated oral sheet (A5) and the corneal (B5) and oral mucosa (D5), whereas cornea-specific keratin-12 immunostaining was not found in the cultivated oral epithelial cells (A6) or in normal conjunctival (C6) and oral epithelial cells (D6), but was found in normal corneal epithelial cells (B6). Scale bar: (A-C) 100 μm; (D) 200 μm.

DISCUSSION

Severe ocular surface diseases such as SJS and OCP are some of the most challenging problems that the clinician faces today. Conventional management is generally unsatisfactory, and the long-term ocular consequences of these conditions are devastating. In the past 10 years, surgical reconstruction of the ocular surface has been greatly advanced by the introduction of limbal epithelial transplantation, AM transplantation, and cultivated corneal epithelial transplantation.[12-27] We have since adopted these procedures for clinical use in severe ocular surface diseases and have achieved successful ocular surface reconstruction.[24,25] However, we normally used grafts transplanted from donors (allografts), putting the recipients at risk of rejection and necessitating the use of prolonged immunosuppression. Although it has been reported that transplantation of the patient's own limbal epithelial cells (autograft) is a simple and effective method of reconstructing the corneal surface with unilateral severe ocular surface disease,[19,20] we normally cannot perform this procedure because diseases such as SJS and OCP involved complete loss of corneal epithelial stem cells and conjunctival keratinization in both eyes. The problem of allograft rejection is the main reason that we decided to develop a new method of autologous mucosal epithelium transplantation for corneal surface reconstruction.

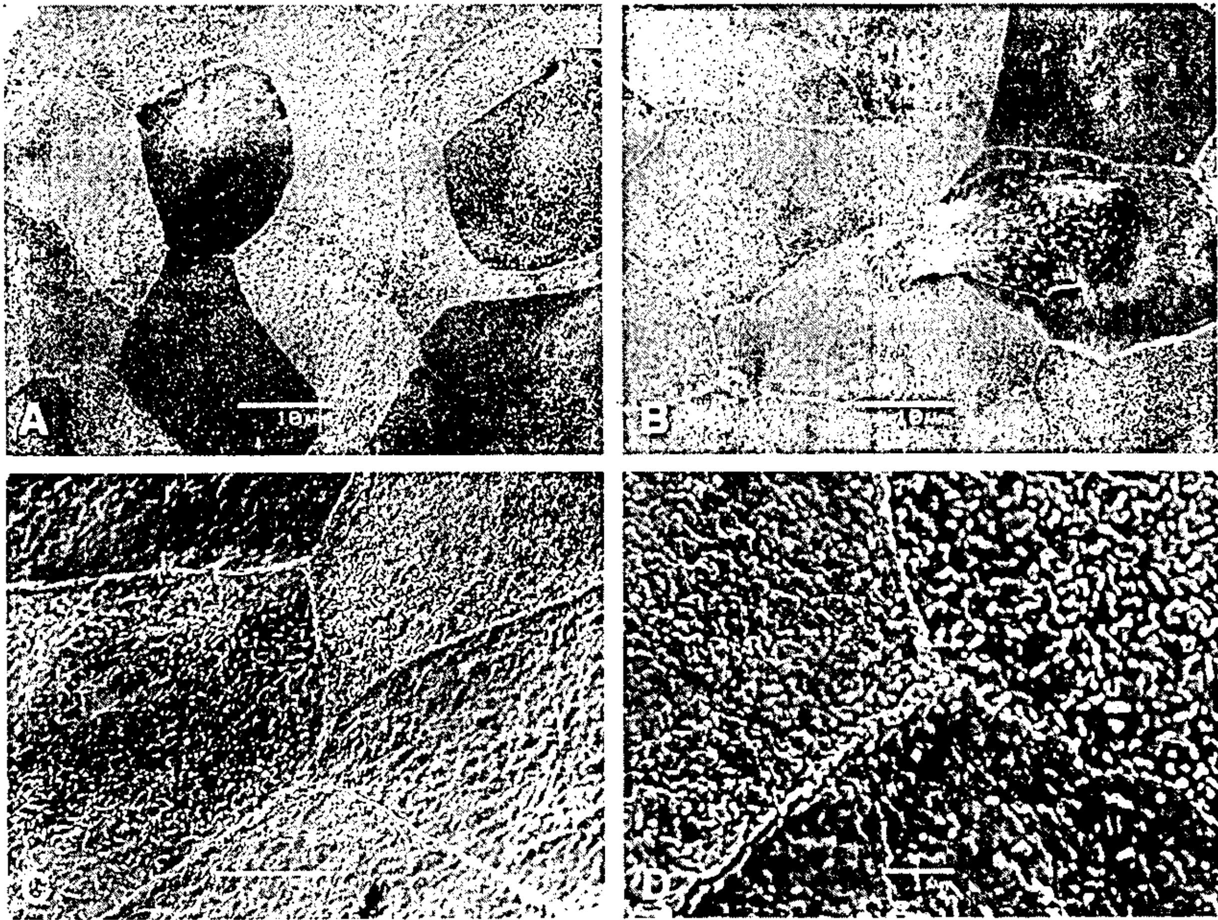

FIGURE 4. Scanning electron micrographs of cultivated mucosal epithelial cells on denuded AM at low magnification (A, B) and at high magnification (C, D). The cells appeared healthy and well formed with distinct cell boundaries (A). In places, desquamating cells were also found (B). The cells appeared to be in good condition and were closely attached to each other with tightly fitting cell junctions (C). The apical surface of the cells was covered with microvilli (D).

In the field of oral surgery, many attempts have been made to reconstruct the oral cavity by skin grafting, oral mucosal grafting, and transplantation of cultured oral mucosal sheets.[30-35] Epithelial cells isolated from the oral mucosa are generally thought to be at a lower stage of differentiation than skin keratinocytes and offer the following potential advantages: short cell turnover time with resultant short culture time requirement and long-term maintenance under culture conditions without keratinization.[36,37] Moreover, the oral mucosa is an ideal location for tissue biopsy, because the resultant scar is inconspicuous. These characteristics suggest that oral epithelial cells could be an ideal substitute for corneal epithelial cells for use in ocular surface reconstruction.

Cultivated corneal epithelial transplantation has long been a dream of ophthalmologists. In recent years, we have successfully developed a surgical system using cultivated corneal epithelial transplantation.[22-27] Our system for culturing is unique in several important respects, which include the use of denuded AM as a carrier and a 3T3 fibroblast layer to assist epithelial cell growth.[23] We have also discovered that air-lifting is crucial for the correct formation of epithelial tight junctions and epithelial cell stratification.[27] We have adopted this system for culturing oral epithelial cells (with some modifications) and have successfully generated confluent cultures of cells, on devitalized human AM, which had been expanded ex vivo from biopsy-derived rabbit oral mucosal tissue. The superficial cells of the cultivated oral cell layer had nuclei, indicating that they were indeed nonkeratinized mucosal epithelium.

Cytokeratins play an important structural and protective role in maintaining the integrity of the epithelium of the anterior segment of the eye.[38-41] In vivo, cytokeratin filament systems are composed of type 1 (neutral-basic) and type 2 (acidic) obligate heterodimers that exist as specific pairs.[41] Defined subsets of individual cytokeratin pairs are characteristically expressed, depending on epithelial cell tissue type and level of differentiation.[42-44] We used immunohistochemistry in this study to demonstrate that the keratin-1 and -10 pair, which is involved in the physiological keratinization process in the epidermis, is not expressed in any layers of the cultivated oral epithelial sheet or in corneal, conjunctival, and oral epithelial cells. We also found that the keratin-4 and -13 pair, which is observed in nonkeratinized, stratified epithelia, is expressed in the superficial and intermediate layers of the cultivated oral epithelial cells, with no discernible immunostaining in the basal cell layers. These keratins were also observed in the conjunctival and oral epithelial cells. These results lead us to believe that the oral epithelial cells cultivated on AM have the characteristics of nonkeratinized mucosa, not of keratinized mucosa. We also observed the general absence of other keratinization-related proteins, such as involucrin and filaggrin (data not shown), which supports our conclusions. Immunohistochemical examination revealed no cornea-specific expression of keratin-12 in any layers of the cultivated oral epithelial sheets or in conjunctival and oral epithelial cells, whereas cornea-specific keratin-3 was expressed in all epithelial layers of the cultivated oral epithelial sheet and oral mu-