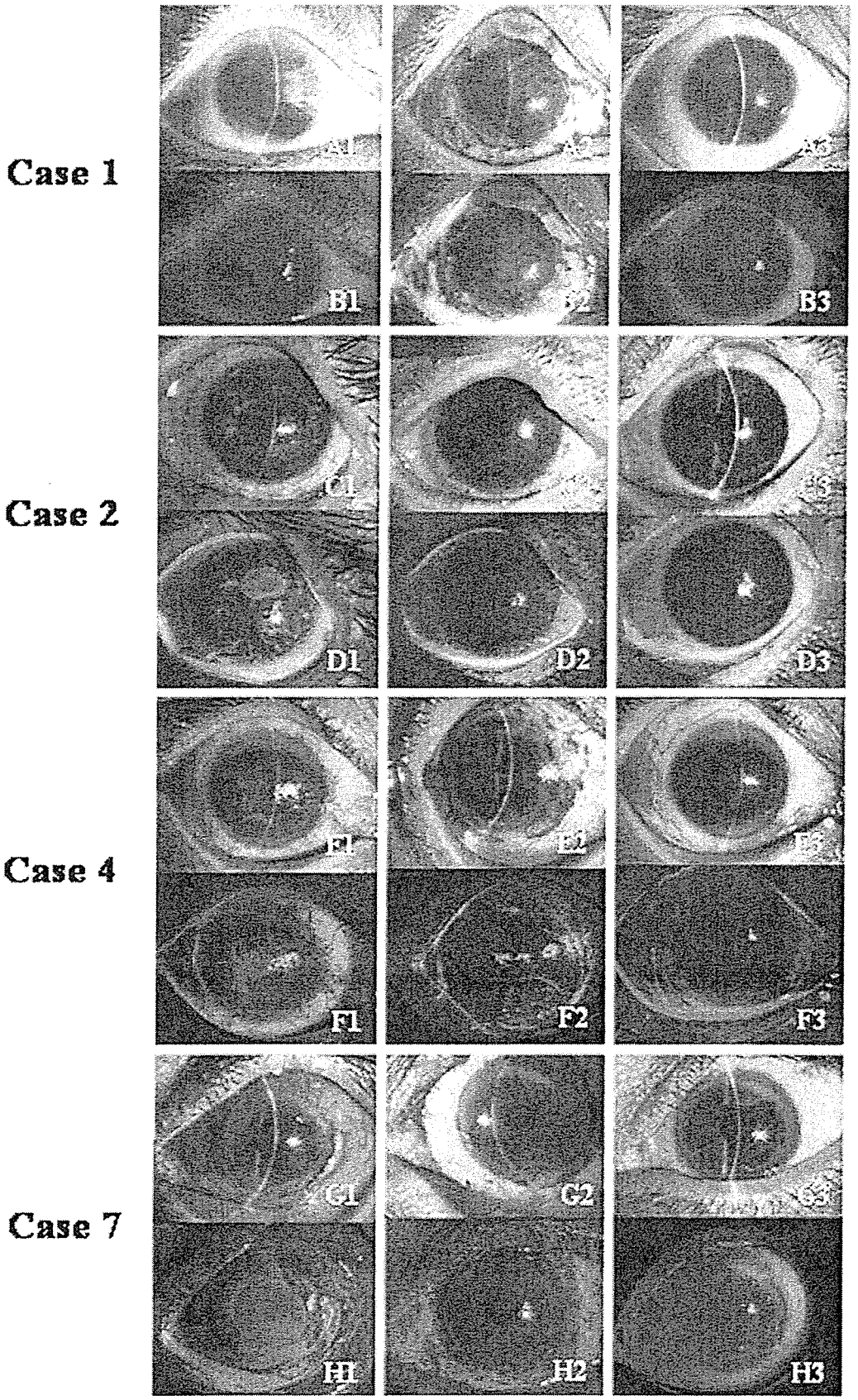
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could be achieved within 2 to 5 days. In this study, we performed ocular surface reconstruction by transplanting allogeneic or autologous AS-derived cultivated corneal epithelial cells to evaluate their efficacy in the treatment of severe ocular surface disease. We found that epithelial cells cultivated using AS could survive and completely epithelialize the cornea within 2 to 5 days after transplantation. The corneal surface of all eyes in the current study remained reasonably clear and smooth, and the entire corneal surface was covered with transplanted epithelium at the last follow-up visit. Although this was a noncomparative clinical study, based on our previous clinical experience, we could find no significant differences between the clinical results of AS- and FBS-derived corneal epithelial transplantation, suggesting that the AS culture system is a safe and effective procedure for ocular surface reconstruction in patients with severe ocular surface disease.

To increase the likelihood of long-term tissue regeneration, it is important that stem or progenitor cells are retained in the cultivated corneal epithelial sheet, as these cells have greater proliferative potential. There is currently no available direct method for determining the exact proportion of stem cells in transplanted tissue equivalents. The ability of transplanted cells to continue to regenerate the ocular surface after prolonged periods is an indirect indicator of the long-term proliferative potential of transplanted cells. Based on the positive clinical findings and the stability of the ocular surface in our patients, up to the longest follow-up period of 20 months, it may be reasonable to assume that cells possessing long-term regenerative potential were present in the original transplanted corneal epithelial sheet. This would suggest that AS was able to maintain and possibly even support the proliferation of stem or progenitor cells in the culture process. This is an important issue that is the subject of ongoing research.

In conclusion, this is the first study that demonstrates the use of AS-derived cultivated corneal epithelial transplantation for ocular surface reconstruction in the treatment of severe limbal stem cell deficiency. The use of AS as an alternative to FBS offers significant advantages because it helps to eliminate the use of animal material from the culture process, thereby reducing 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. Although the long-term survival of these grafts has yet to be determined, these findings are an important advancement in the field of ocular surface bioengineering for the treatment of patients with severe ocular surface disease.

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Figure 2. Before transplantation, all eyes manifested severe destruction of the ocular surface with limbal stem cell deficiency (**left column**). Two to 4 days after transplantation, the corneal surface of all treated eyes was clear and smooth; fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (**middle column**). The transplanted epithelial sheet was surrounded 360 degrees by conjunctival epithelial defects, indicating that there was no contamination of the host conjunctival epithelium. Slit-lamp examination showed that conjunctival fibrosis was successfully suppressed in all patients and the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects at the last follow-up visit (**right column**).

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

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

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

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

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

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

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

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

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

METHODS

Human Subjects

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

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Primary Culture of HCjE Cells

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

Conjunctival Epithelium Transplantation onto Rabbit Corneas

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

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

Slit Lamp Examination

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

Tissue Preparation

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

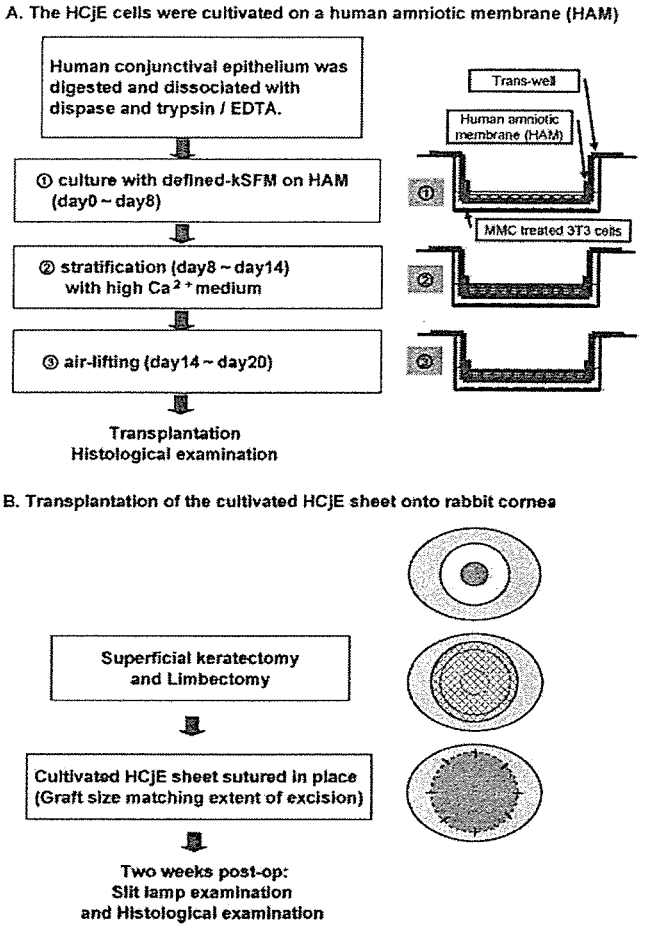

FIGURE 1. Cell culture of HCjE and transplantation into rabbit cornea.

Immunostaining and Light-Microscopic Analysis

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

Transmission Electron Microscopic Examination

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

TABLE 1. Antibodies Used in the Study

Group	Antigen	Dilution	Type of Antibody	Immunized Animal	Company*	Annotation
Putative stem cell markers	ABCG2	×40	(Mo)	M	Kamiya	ATP-binding cassette transporter
	p63	×100	(Mo)	M	Santa Cruz	p53 homologous protein
Adhesion molecule	Laminin5	×100	(Mo)	M	Chemicon	Hemidesmosome component protein
	Integrin α6	×100	(Mo)	M	Cymbus	Hemidesmosome component protein
	Integrin β4	×100	(Mo)	M	Chemicon	Hemidesmosome component protein
	Desmoplakin	×1	(Mo)	M	Progen	Desmosome component protein
Nuclei	Human nuclei	×30	(Mo)	M	Chemicon	Possible to distinguish human cells from other animal cells
Cytokeratin	CK3	×50	(Mo)	M	Progen	Major cytokeratin in corneal epithelium
	CK4	×100	(Mo)	M	Novocastra	Major cytokeratin in nonkeratinizing mucosal epithelium
	CK12	×100	(Po)	G	Santa Cruz	Major cytokeratin in corneal epithelium
	CK13	×200	(Mo)	M	Novocastra	Major cytokeratin in nonkeratinizing mucosal epithelium
Mutin	MUC4	×50	(Mo)	M	Zymed	A membrane-bound mucin
	MUC5AC	×100	(Mo)	M	Novocastra	Secreted mucin/goblet cell mucin

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

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

RESULTS

Analysis of HCjE Sheets

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

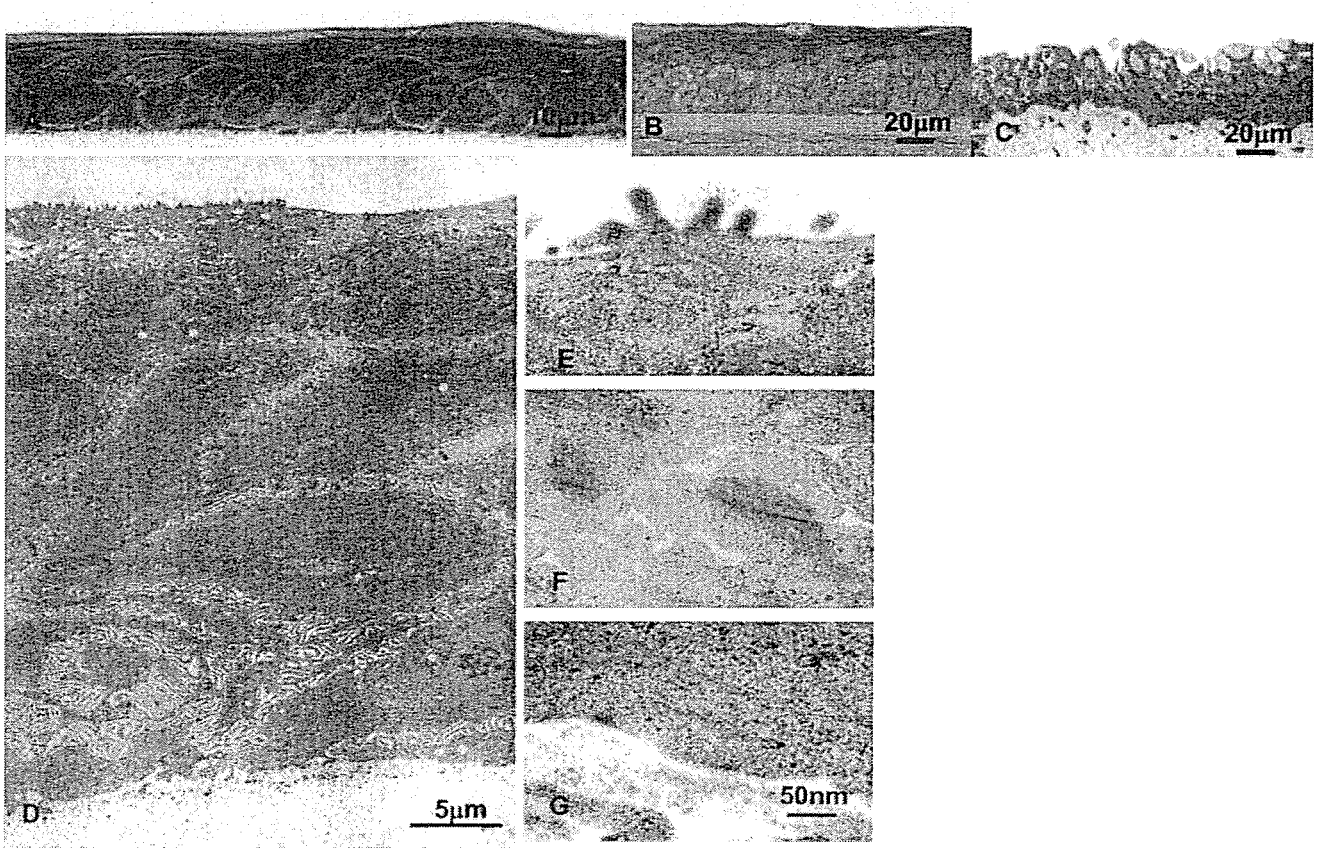

FIGURE 2. Histologic examination of HCjE cells grown on human amniotic membrane. Cultivated human conjunctival epithelium and in vivo corneal- and conjunctival epithelium were examined by light microscopy (A–C: semithin section stained with toluidine blue) or transmission electron microscopy (D–G). The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

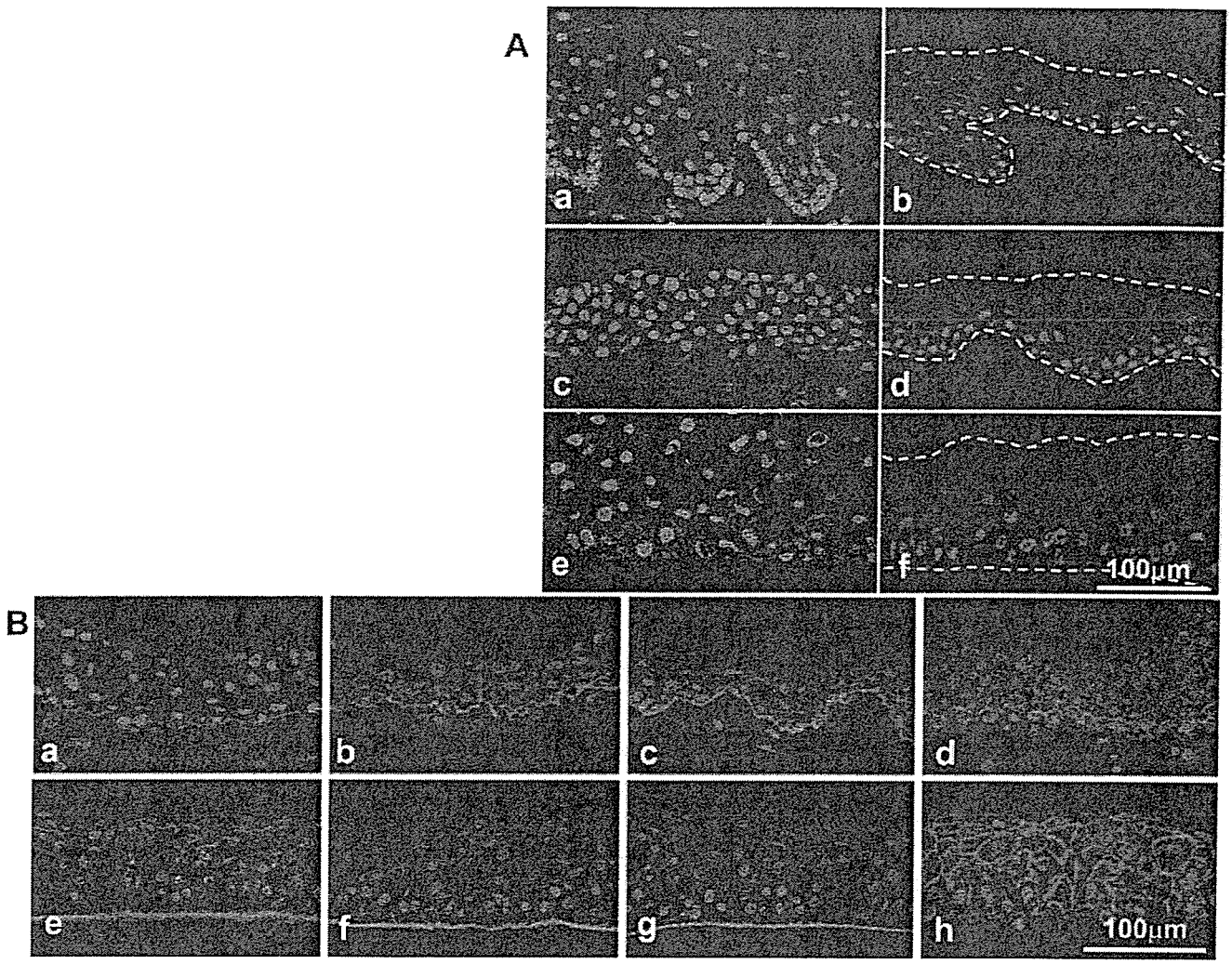

FIGURE 3. Expression of putative markers for stem/progenitor cells and epithelial adhesion molecules in the cultivated HCjE. (**A**) In vivo human limbal epithelium (**Aa**, **Ab**), in vivo HCjE (**Ac**, **Ad**), and cultivated HCjE (**Ae**, **Af**) were immunostained (*green*) with ABCG2 (**Aa**, **Ac**, **Ae**) or p63 (**Ab**, **Ad**, **Af**) and counterstained with propidium iodide (*red*). (**B**) In vivo HCjE (**Ba**, **Bb**, **Bc**, **Bd**) and cultivated HCjE (**Be**, **Bf**, **Bg**, **Bh**) were immunostained (*green*) with laminin5 (**Ba**, **Be**), integrin α6 (**Bb**, **Bf**), integrin β4 (**Bc**, **Bg**), or desmoplakin (**Bd**, **Bh**) and counterstained with propidium iodide (*red*).

Frozen sections of in vivo ocular tissues and cultivated HCjE were subjected to indirect immunostaining analysis. The basal cells of the cultivated HCjE sheets expressed the putative stem cell markers ABCG2 and p63 (Fig. 3Aa–Af); their expression patterns were almost identical with those of in vivo limbal epithelium. The hemidesmosome component proteins laminin 5 and integrin α6β4 were restricted to the interface between the basal cells and the AM. Desmoplakin, a desmosome-associated protein, was expressed at cell–cell borders. These expression patterns were almost identical with those of in vivo HCjE (Fig. 3Bg–Bn).

Transplantation of Cultivated HCjE Sheets

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

Histologic and EM Appearance of the Engrafted Conjunctival Epithelium

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

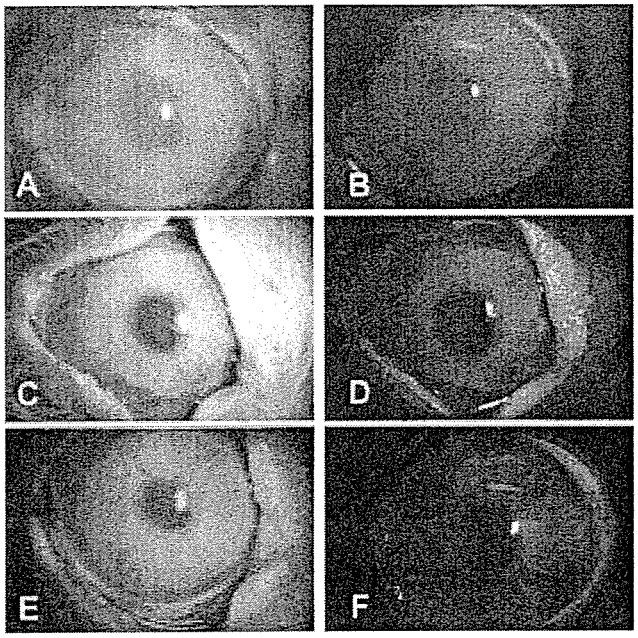

FIGURE 4. HCjE grafted onto the denuded rabbit cornea. The engrafted HCjE was inspected just after transplantation (A, B) and 4 (C, D) and 14 days (E, F) after transplantation. The engrafted HCjE was devoid of epithelial defects at all observation points.

Immunohistochemistry

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

Discussion

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

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

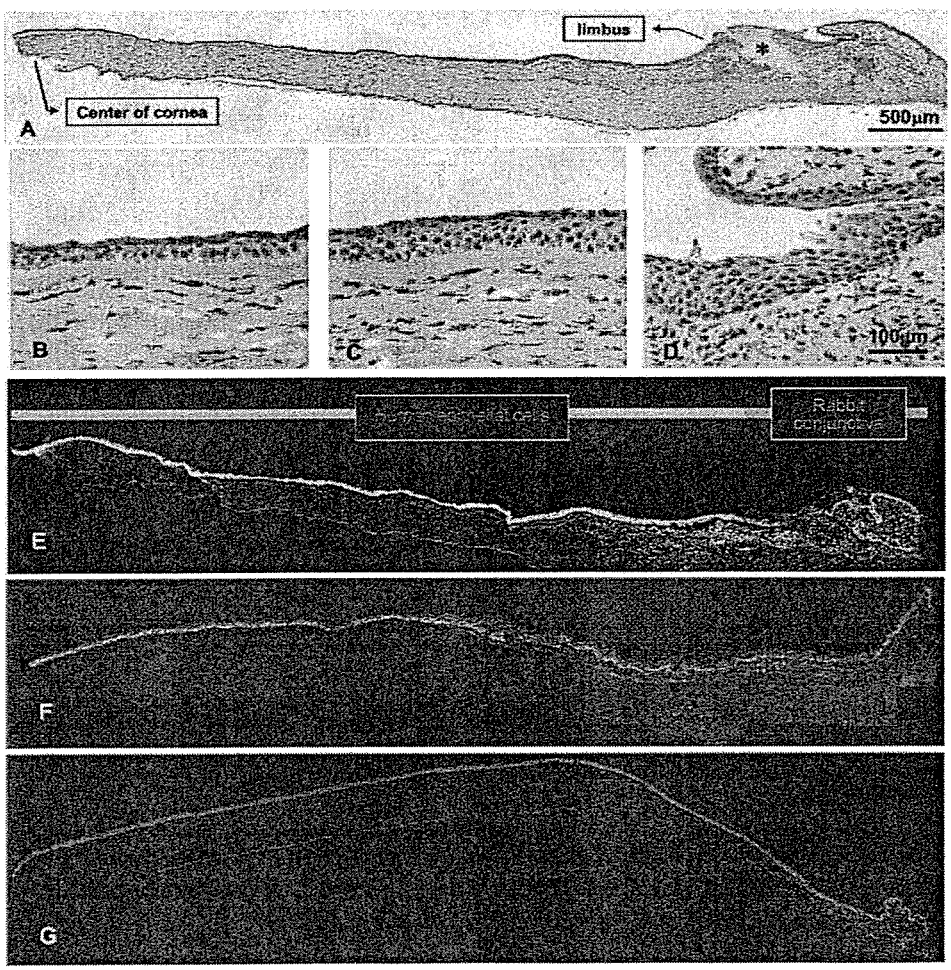

FIGURE 5. Light microscopy of the engrafted tissue and distribution of human epithelial cells on the graft. At 2 weeks, the engrafted epithelium demonstrated five to six layers of stratified squamous epithelium similar to normal corneal epithelium (A–C). (D) Conjunctiva of the recipient. No goblet cells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

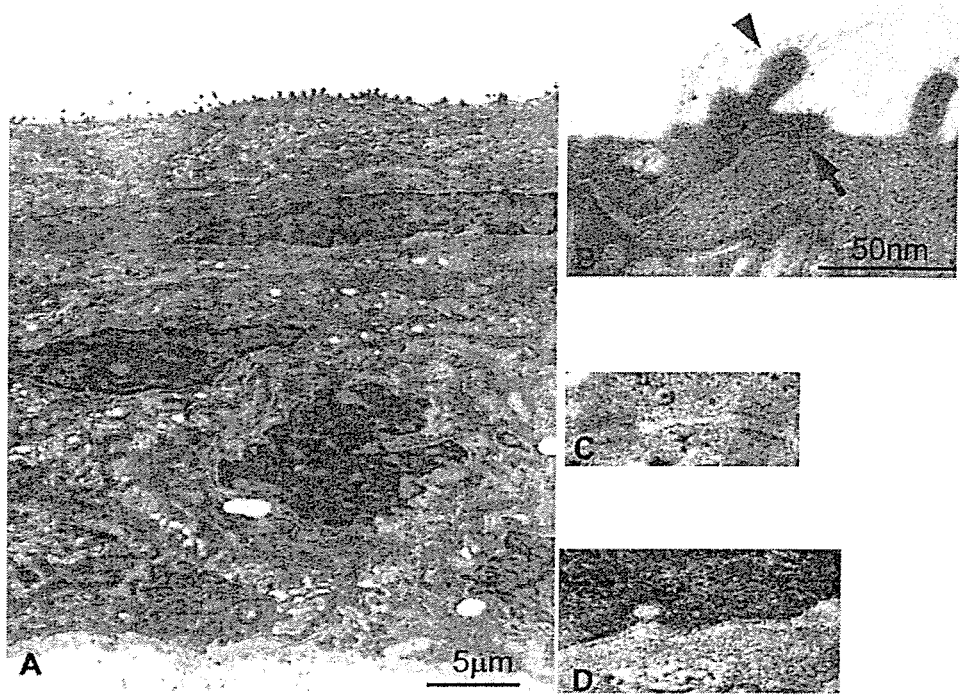

FIGURE 6. Transmission electron microscopy of the engrafted tissue. (A) Transmission electron microscopy at low magnification; (B) microvilli (*arrowhead*), tight junction (*arrow*); (C) desmosome (*arrow*); (D) hemidesmosome.

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

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

For the transplantation of HCjE to be successful, the cultivated sheet must possess structural integrity. The normal corneal epithelium features desmosomes at the cell–cell interface, and their presence contributes to its structural integrity. Hemidesmosomes at the basal cell surface serve to attach the basal cells to the underlying basement membrane. We demonstrated that desmosome-associated (desmoplakin),[28] hemidesmosome-associated (integrin $\alpha 6\beta 4$),[29] and basement membrane-associated (laminin 5)[30,31] proteins were present in the cultivated HCjE sheets. Furthermore, as in the corneal limbus, basal cells in the HCjE sheets expressed the putative stem cell markers ABCG2 and p63,[32,33] suggesting that they possess the structural and regenerative characteristics of corneal and limbal epithelium.[34]

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

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

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

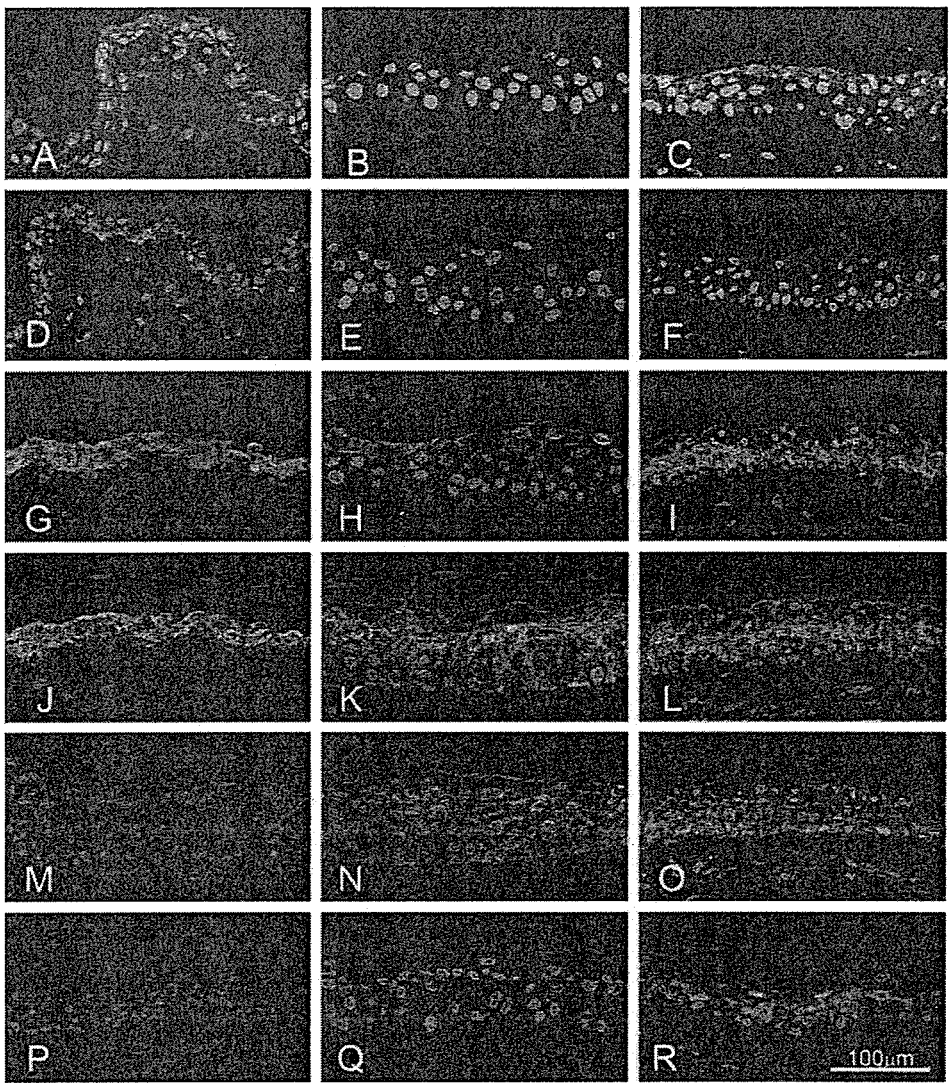

FIGURE 7. Immunohistochemical examination of the engrafted epithelium. In vivo HCjE (**A**, **D**, **G**, **J**, **M**, **P**), cultivated HCjE (**B**, **E**, **H**, **K**, **N**, **Q**), and engrafted epithelium (**C**, **F**, **I**, **L**, **O**, **R**) were immunostained (*green*) with MUC4 (**A**–**C**), MUC5AC (**D**–**F**), CK4 (**G**–**I**), CK13 (**J**–**L**), CK3 (**M**–**O**), or CK12 (**P**–**R**) and counterstained with propidium iodide (*red*).

ment. Because we were dealing with xenotransplantation, one of the limitations of this study is the short follow-up period of 14 days. With more prolonged follow-up, it may be that some conjunctival cells would differentiate into goblet cells and that progressive conjunctivalization and neovascularization would occur. More long-term studies are needed to investigate some of these questions.

In summary, ours is the first report that clearly demonstrates the potential of cultivated HCjE as an alternative tissue source for replacement of the corneal epithelium. Our animal study is a step toward the eventual transplantation of autologous cultivated HCjE to treat patients with ocular surface disorders, and studies are ongoing to resolve outstanding issues.

Acknowledgments

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Autologous Serum–Derived Cultivated Oral Epithelial Transplants for Severe Ocular Surface Disease

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Objective: To evaluate the use of autologous serum (AS)–derived cultivated oral epithelial transplants for the treatment of severe ocular surface disease.

Methods: We used AS from 10 patients with severe ocular surface disease and total limbal stem cell deficiency to develop autologous cultivated oral epithelial equivalents. These were compared with epithelial equivalents derived from conventional fetal bovine serum–supplemented medium. Surgery involved removal of the corneal pannus and surrounding diseased tissue and transplantation of the AS-derived epithelial equivalents. The oral equivalents were analyzed by review of histologic and immunohistochemical findings.

Results: Oral epithelial sheets cultivated in AS- and fetal bovine serum–supplemented media were similar in morphology, and both formed basement membrane assembly proteins important for maintaining graft integrity. Complete corneal epithelialization was achieved within 2 to 5 days postoperatively. The ocular surface remained stable without major complications in all eyes during a mean±SD follow-up of 12.6±3.9 months. The visual acuity improved by more than 2 lines in 9 of 10 eyes, with transplanted oral epithelium surviving up to 19 months.

Conclusion: The successful use of an AS-derived oral epithelial equivalent to treat severe ocular surface disease represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation.

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SEVERE OCULAR SURFACE DISease (OSD) arising from conditions such as Stevens-Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury is a potentially serious blinding condition that represents a major clinical challenge. In such cases, destruction of the corneal epithelial stem cells located at the limbus results in conjunctival invasion, corneal neovascularization, chronic inflammation, and stromal scarring.[1-3] These conditions respond poorly to conventional corneal transplantation. Corneal epithelial replacement by means of keratoepithelioplasty and limbal transplantation has been used to treat these severely damaged eyes.[4-6] However, a significant proportion of these allografts ultimately fail, resulting in visual loss.[7-9] In recent years, bioengineered corneal epithelial equivalents, developed from the ex vivo expansion of limbal stem cells, have been used to treat severe limbal stem cell deficiency, with promising results.[10-17]

Most of the previous reports on cultivated corneal epithelial transplantation used allogeneic tissue because many of these severe conditions have bilateral eye involvement.[10,12-17] Fetal bovine serum (FBS)–supplemented medium remains the medium of choice in the culture process.[11-20] We previously demonstrated that autologous oral epithelial transplantation for treating severe OSD is particularly useful in bilateral disease where healthy tissue is lacking.[18,19] However, the use of FBS may be associated with the risk of transmission of zoonotic infection (eg, bovine spongiform encephalitis) and other unknown pathogens. Because bovine spongiform encephalitis cannot be detected by any known in vitro methods, this use of bovine products is a major health concern in many parts of the world. A group from our institution previously showed that human serum was able to support epithelial cell proliferation,[21] which raises the possibility of using the patient's own serum as an alternative to FBS in the culture process. The use of autolo-

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gous serum (AS) is advantageous because it eliminates the need for bovine material and reduces the risk of disease transmission.

In this study, we compared the efficacy of AS supplementation with that of conventional FBS supplementation in developing cultivated oral epithelial equivalents and evaluated the use of AS-derived cultivated oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. We describe the successful clinical use of bioengineered ocular surface equivalents that are derived almost entirely from autologous tissue and material. This study has important clinical implications and represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation because it reduces the risks of transmitted infection and allograft rejection, as well as the need for long-term immunosuppression.

METHODS

SUBJECTS

All experimental procedures and clinical applications were approved by the institutional review board for human studies of the 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.

The study included 10 eyes from 10 patients with severe OSD who underwent autologous cultivated oral epithelial transplantation at our hospital between April 1, 2004, and May 30, 2005. The patients consisted of 7 men and 3 women; their ages ranged from 19 to 75 years (mean±SD age, 57.1±18.9 years). The preoperative diagnosis was SJS in 7 patients, thermal injury in 1, chemical injury in 1, and ocular cicatricial pemphigoid in 1. Preoperatively, all 10 eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. These patients demonstrated a reasonable reflex tear function and tear meniscus level. All patients were followed up for a minimum of 6 months after transplantation, with the longest follow-up being 19 months.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SOLUBLE Fas LIGAND

Previous reports have shown that a high concentration of soluble Fas ligand (sFasL) at the onset of SJS may play a crucial role in keratinocyte apoptosis.[22] We analyzed the serum sFasL levels of the patients with severe OSD to determine whether AS could be used safely as a cell culture supplement. We determined the concentrations of sFasL by means of an sFasL enzyme-linked immunosorbent assay kit (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan), following the manufacturer's protocol, with the reaction measured at 450 nm. The limit of detection was 100 pg/mL. Each individual sample was analyzed in duplicate. Human serum samples from healthy age-matched volunteers were used as control samples.

CULTIVATION OF ORAL EPITHELIAL SHEET

Under aseptic conditions, AS was obtained from each patient by venesection at the antecubital fossa. We collected 30 mL of blood in a sterile container and centrifuged and filtered the sample, yielding a purified serum sample of approximately 10 mL. Each patient's serum sample was stored in sterile tubes at −30°C before use.

PREPARATION OF AUTOLOGOUS CULTIVATED ORAL EPITHELIAL EQUIVALENT

The presence of healthy oral mucosa was first confirmed by a dentist before biopsy. An oral mucosal biopsy specimen, 2 to 3 mm^2, was obtained with the patient under local anesthesia. The submucosal connective tissue was first carefully removed with scissors. The oral epithelium was then incubated at 37°C for 1 hour with 1.2-IU dispase, followed by treatment with a solution of 0.05% trypsin and EDTA for 10 minutes to separate the cells. The resultant oral epithelial cells (1×10^5/mL) were then seeded onto denuded amniotic membranes spread on the bottom of culture inserts and cocultured with mitomycin-inactivated 3T3 fibroblasts.[18,19] The culture medium consisted of defined keratinocyte growth medium (ArBlast Co Ltd, Kobe, Japan) supplemented with 5% AS and insulin (5 μg/mL), cholera toxin (0.1 nmol/L), human recombinant epidermal growth factor (10 ng/mL), and a mixture of penicillin and streptomycin (50 IU/mL).[17]

The cultured cells were submerged in the medium for 2 weeks and then airlifted for 1 to 2 days by lowering the medium level. Cultures were incubated at 37°C with 5% carbon dioxide and 95% air, with the medium changed every day. To compare the use of the AS-supplemented medium with that of the conventional FBS-supplemented medium, we also cultivated the patient's oral epithelial cells in FBS-supplemented medium. We compared the morphological and immunohistochemical results of the oral epithelial equivalents prepared in AS-supplemented medium with the corresponding oral epithelial equivalents prepared in FBS-supplemented medium.

SURGICAL PROCEDURE

We performed a 360° conjunctival peritomy 3 mm from the limbus, and removed all perilimbal scarred or inflamed subconjunctival tissue to the bare sclera. The corneal pannus was completely removed by blunt dissection or superficial keratectomy using surgical scissors or a blade. We then treated the residual subconjunctival tissue with 0.04% mitomycin for 5 minutes, followed by vigorous repeated washing with isotonic sodium chloride solution.[13,14] The cultivated autologous oral epithelial sheet was cut from the culture insert using a 19-mm diameter trephine, transferred over the corneal surface, and secured in place with 10-0 nylon sutures at the limbus. In patients with more extensive disease such as symblepharon formation or fornical shortening, ocular surface reconstruction was performed by transplanting an additional amniotic membrane over the surrounding scleral surface or fornix, securing it in place with 10-0 nylon sutures, and reconstructing the rest of the ocular surface. The integrity of the cultivated epithelium was confirmed by fluorescein staining results at the end of surgery, and the ocular surface was protected with a medical-use bandage contact lens.

Postoperatively, eyedrops consisting of 0.3% ofloxacin and 0.1% dexamethasone sodium phosphate were instilled 4 times a day. The eyedrop therapy was tapered to a maintenance dosage of 2 to 3 times a day after 2 to 3 months, depending on the severity of inflammation. Oral betamethasone sodium phosphate (1 mg/d) and cyclosporine (100 mg/d) were administered to reduce inflammation, and dosages were tapered and stopped 1 month postoperatively. Patients were followed up with slitlamp examination, fluorescein staining, and photographic documentation.

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATION

A representative piece of oral epithelial equivalent was sent for histological and immunohistochemical analyses. Cryostat sections (7-μm thick) were placed on gelatin-coated slides and air-dried. Morphological analysis was performed by staining with hematoxylin-eosin. Immunohistochemical studies of tissue-specific keratins and cell junction specialization-related proteins were performed as previously described.[23,24] Tissue sections were incubated for 1 hour with primary antibodies to keratin 3 (Progen Biotechnik GmbH, Heidelberg, Germany), keratin 4 (Novocastra, Newcastle, England), keratin 13 (Novocastra), ZO-1 (Zymed Laboratories, Inc, South San Francisco, Calif), desmoplakin (Progen), integrin α6 (Chemicon International, Temecula, Calif), laminin 5 (Chemicon International), and collagen IV (MP Biomedicals, Irvine, Calif). This was followed by incubation with the appropriate secondary antibodies, fluorescein isothiocyanate–conjugated donkey anti–mouse IgG and fluorescein isothiocyanate–conjugated goat anti–rabbit IgG (Molecular Probes; Eugene, Ore). The sections were were covered with antifading mounting medium containing propidium iodide (Vectashield; Vector Laboratories, Burlingame, Calif) and were examined by confocal microscopy (Fluoview; Olympus Corp, Tokyo, Japan).

RESULTS

SERUM sFasL LEVEL IN SEVERE OSD

The serum levels of sFasL were undetectable in the 10 patients with severe OSD. These results were similar to the control group of 10 healthy volunteers.

MORPHOLOGY OF CULTIVATED ORAL EPITHELIAL EQUIVALENT

Cultivated oral epithelial cells proliferated on the denuded amniotic membranes and formed a confluent sheet of epithelial cells within 5 to 8 days. After 2 weeks, the cultivated oral epithelium consisted of 4 to 6 layers of cells, with a well-conserved basal layer consisting of cuboidal cells and progressively flattened cells superficially. Results of the histological examination showed that the structure and organization of the oral epithelial sheets cultivated in AS- and FBS-supplemented media were similar (**Figure 1**A).

The expression patterns of tissue-specific keratins and cell junction specialization-related proteins were similar in AS- and FBS-derived oral epithelial equivalents. In the AS- and FBS-supplemented cultures, keratin 4 was expressed in the superficial and upper half of the intermediate layers (Figure 1B). Keratin 13 was expressed throughout the epithelium (Figure 1C). Keratin 3, a cornea-associated differentiation marker, was expressed in all cell layers (Figure 1D). The tight junction–related protein ZO-1 was expressed in the apical surfaces of cultivated epithelium (**Figure 2**A). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (Figure 2B). Basement membrane assembly proteins, such as integrin α6, laminin 5, and collagen IV, showed linear positive staining on the basement membrane side of the epithelium (Figure 2C-E). The expression patterns of all of these proteins were similar in oral epithelial sheets cultivated in the AS- and FBS-supplemented culture media.

CLINICAL RESULTS

The clinical data and surgical outcomes of the 10 patients are summarized in the **Table**. The mean ± SD follow-up period was 12.6±3.9 months, with the longest follow-up being 19 months. Before transplantation, all eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. Two to 5 days after transplantation, the corneal surfaces of all treated eyes were clear and smooth, and fluorescein staining confirmed that they were entirely covered by the cultivated oral epithelium. The presence of an initial intervening nonepithelialized area between the host conjunctiva and the transplanted oral epithelium confirmed that epithelialization did not arise from the adjacent host conjunctiva.

Successful engraftment was achieved in all patients, with no sloughing off of any of the grafts. Ocular inflammation was found to subside rapidly after surgery in all patients. Slitlamp examination showed that conjunctival fibrosis was successfully suppressed in all patients, with no conjunctival invasion on the corneal surface throughout the follow-up. At the last follow-up visit, the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects. The clinical progress of 2 representative patients with total limbal stem cell deficiency arising from SJS (patient 3) and ocular cicatricial pemphigoid (patient 7) is shown in **Figure 3** and **Figure 4**.

Nine (90%) of the 10 eyes were restored to good vision, with visual acuity improving by 2 lines or more at the last follow-up visit. The ocular surface in the right eye of patient 5 was successfully reconstructed and remained stable; however, residual corneal stromal scarring precluded good vision in this eye. All patients experienced a significant improvement in symptoms and a reduction in ocular inflammation compared with their preoperative condition.

There were no significant postoperative complications, and none of the patients developed graft rejection. The right eye of patient 3 developed a small epithelial defect with mild cellular infiltration, suggestive of a low-grade bacterial infection. This was resolved promptly after administration of antibiotic eyedrops consisting of ofloxacin and cefmenoxime hydrochloride. During the follow-up period, 4 eyes developed small epithelial defects that eventually healed over from the adjacent oral mucosal epithelium (**Figure 5**). All of the eyes demonstrated some degree of superficial peripheral corneal neovascularization. This gradually abated with time and did not interfere with vision or cause any postoperative complications.

COMMENT

Cultivated epithelial equivalents have been used for corneal epithelial replacement and regeneration in severe

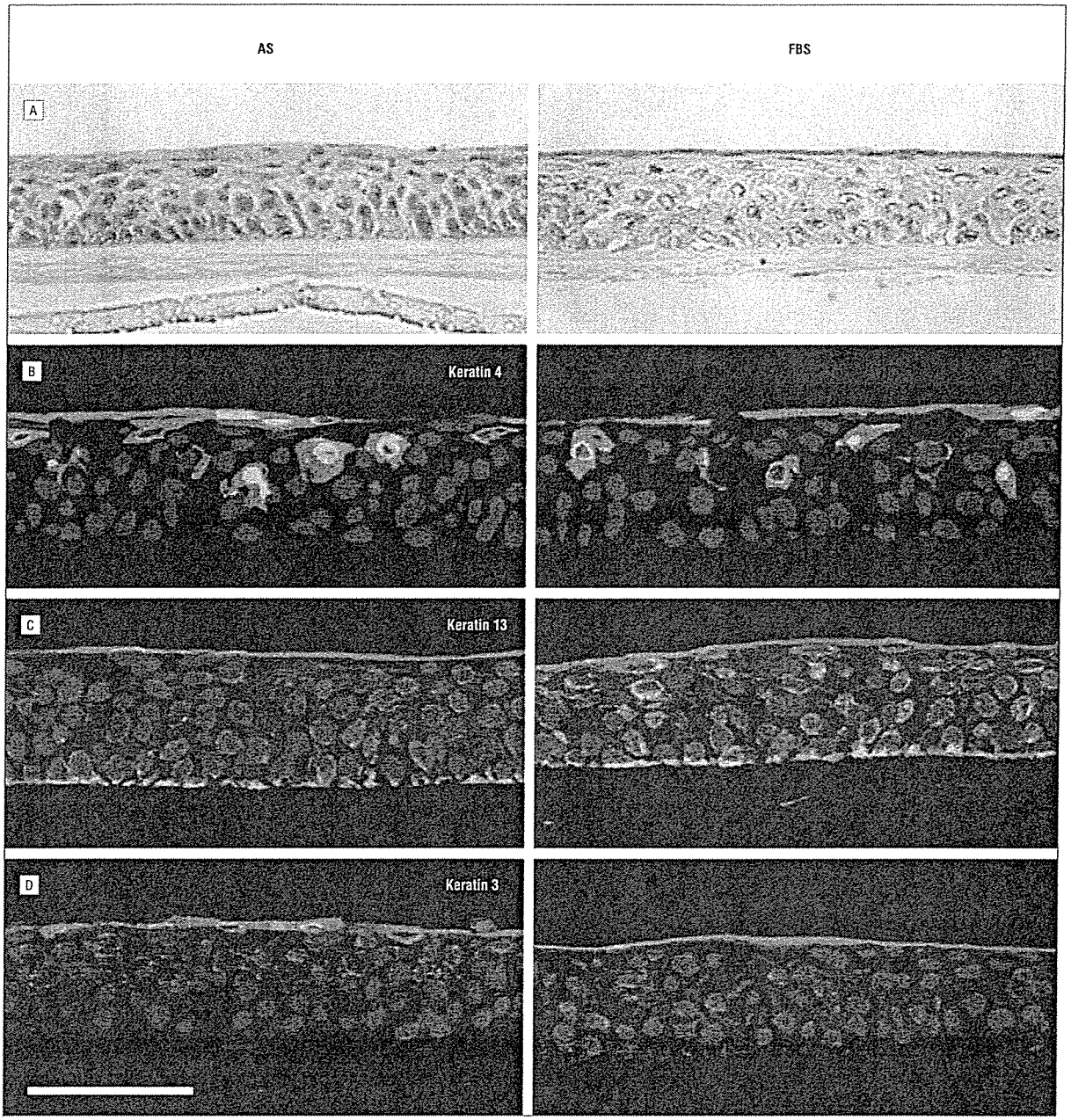

Figure 1. Light microscopy (A) and immunohistochemical findings (B-D) of autologous cultivated oral epithelial sheets. Oral epithelial sheets cultivated in autologous serum–supplemented and fetal bovine serum–supplemented media (left and right sides, respectively) demonstrated a similar histological appearance, with 4 to 6 layers of stratified, well-differentiated cells (A). In both culture systems, the mucosal-specific keratin 4 was expressed in the superficial and upper half of the intermediate layers (B). Keratin 13 was expressed throughout the epithelium (C). Keratin 3 was also expressed in all epithelial cell layers (D). Scale bar indicates 100 μm.

OSD. Fetal bovine serum–supplemented medium remains the most widely used culture system for ocular surface epithelial cell propagation.[11-20,25-29] The ideal culture system for developing transplantable tissue equivalents is one that is safe from disease transmission and maintains the properties of the tissue of origin. We have demonstrated that AS-derived oral epithelial equivalents were similar in structure and organization to those derived from conventional FBS-supplemented cultures. We describe herein the effective use of AS-derived cultivated oral epithelial transplantation for the treatment of severe OSD. The novel approach of using AS as an alternative to FBS in the culture process represents a significant advance in the development of safer, completely autologous bioengineered tissue equivalents for clinical transplantation.

Transplanting autologous eye tissues is possible only if there is sufficient healthy tissue available from the contralateral eye. In most cases of severe OSD, ocular involvement is bilateral. As such, most of the previous

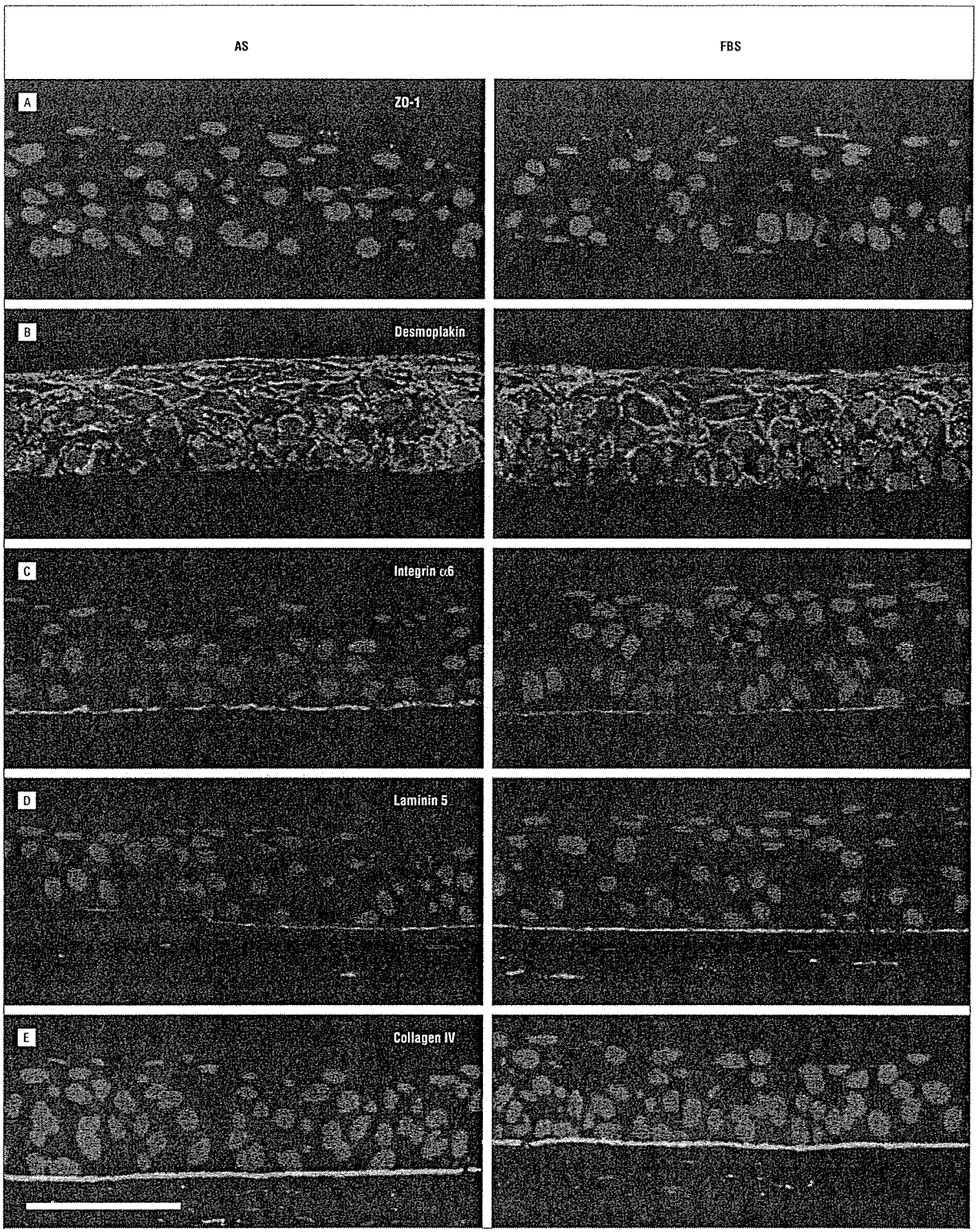

Figure 2. Immunohistochemical findings of cell-to-cell and basement membrane junctional assembly proteins in autologous serum (AS)–supplemented and fetal bovine serum (FBS)–supplemented culture systems (left and right sides, respectively). The ZO-1 protein was expressed at the apical surfaces of the cultivated oral epithelial sheets (A). Desmoplakin, a cell-to-cell junctional component, was expressed in the cell membranes of epithelial cells (B). We noted linear positive staining of integrin α6 (C), laminin 5 (D), and collagen IV (E) on the basement membrane side of the cultivated oral epithelial sheet. The expression patterns of these proteins were similar in cultivated epithelial sheets derived from AS-supplemented and FBS-supplemented culture systems. Scale bar indicates 100 μm.

Table. Clinical Data and Surgical Outcome of Patients*

Patient No./ Sex/Age,y	Disease	Eye	Additional Procedures†	Preoperative Visual Acuity	Postoperative Visual Acuity	Follow-up, mo
1/F/68	SJS	Left	AMT + Phaco/IOL	HM	20/630	19
2/M72	SJS	Left	AMT	HM	20/1000	18
3/F/70	SJS	Right	Phaco/IOL	HM	20/2000	15
4/M/31	Thermal burn	Right	None	20/500	20/63	15
5/M/65	Chemical injury	Right	AMT + Phaco/IOL	20/500	20/500	12
6/M/19	SJS	Right	None	HM	20/50	11
7/M/75	OCP	Left	AMT + Phaco/IOL	HM	20/1000	10
8/F/63	SJS	Left	AMT + Phaco/IOL	HM	20/1000	10
9/M/53	SJS	Left	Phaco/IOL	HM	20/32	8
10/M/57	SJS	Right	AMT	HM	20/1000	8

Abbreviations: AMT, amniotic membrane transplantation; HM, hand motions; OCP, ocular cicatricial pemphigoid; Phaco/IOL, phacoemulsification of cataract and intraocular lens implantation; SJS, Stevens-Johnson syndrome.

*All patients had complete epithelialization within 2 to 5 days.

†All patients underwent removal of the corneal pannus and surrounding diseased tissue and transplantation of autologous serum–derived epithelial equivalent.

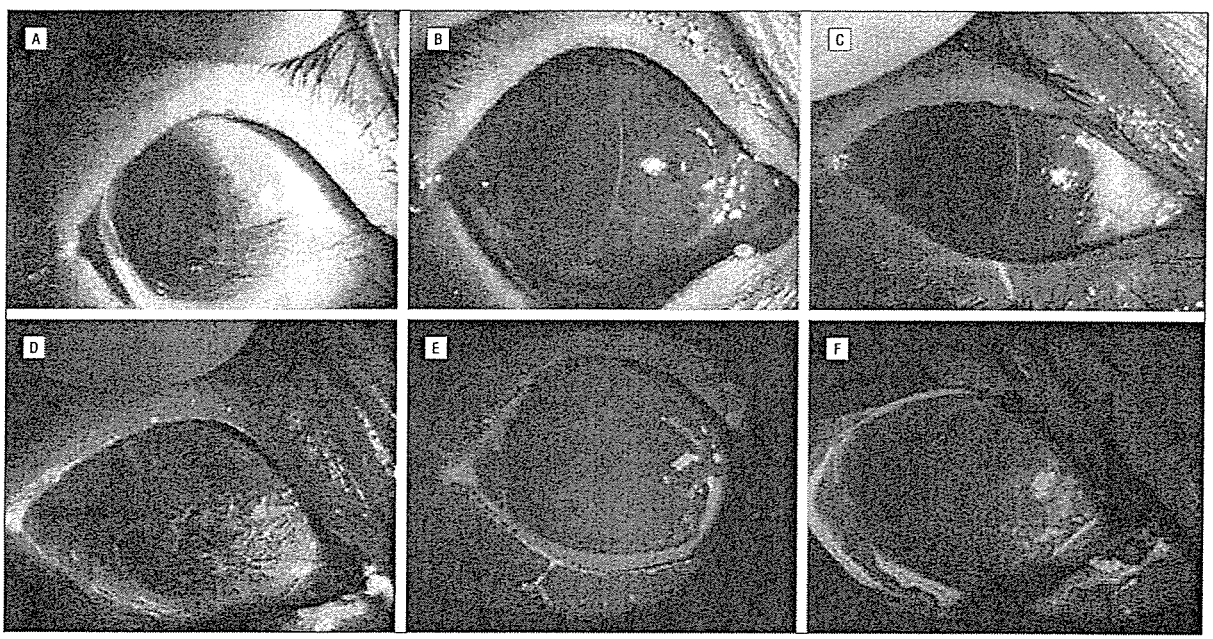

Figure 3. External appearance (A-C) with corresponding fluorescein staining (D-F) in a patient with Stevens-Johnson syndrome and total limbal stem cell deficiency (patient 3). The preoperative appearance (A) demonstrates extensive conjunctivalization, neovascularization, and scarring, with persistent epithelial defects noted on fluorescein staining (D). Two days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). The postoperative appearance at 15 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

reports on cultivated epithelial transplantation used allogeneic tissue.[10,13-17] After allogeneic transplantation in these vascularized and inflamed eyes, patients require long-term medication, such as corticosteroids and immunosuppressive agents, to prevent allograft rejection. In addition, many of these severely damaged eyes require multiple ocular surgical procedures, such as penetrating or lamellar keratoplasty to remove significant corneal scarring, before vision can be satisfactorily restored.[6] Previous use of allogeneic transplantation may predispose these eyes to an increased risk of corneal graft rejection and failure.[30,31] In our study, the use of autologous oral epithelial transplantation for ocular surface epithelialization overcame the problems related to allogeneic transplantation because it helped reduce the risk of graft rejection and the need for long-term medication and immunosuppression. This is particularly important in conditions where multiple reconstructive procedures or transplantations are required for long-term restoration of vision.

Stevens-Johnson syndrome is a major cause of severe OSD, and afflicted patients often have multisystemic involvement. A high concentration of sFasL at the onset of SJS has previously been shown to play a role in keratinocyte apoptosis and in the pathophysiology of the disease.[22] In our study, we found that serum sFasL levels

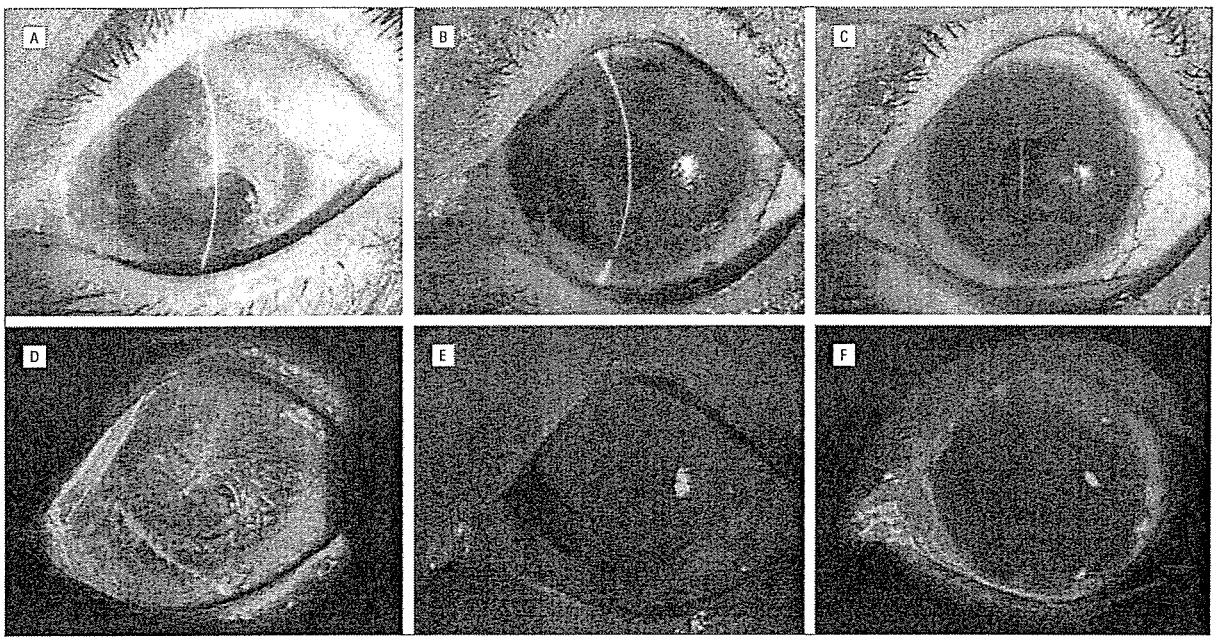

Figure 4. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with ocular cicatricial pemphigoid and severe ocular surface disease with total limbal stem cell deficiency (patient 7). Preoperative appearance demonstrates superior symblepharon formation, extensive conjunctivalization, neovascularization, and scarring of the cornea (A), and severe epitheliopathy is demonstrated on fluorescein staining (D). Five days after transplantation, the corneal surface was clear and smooth (B), and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated epithelium (E). Postoperative appearance at 8 months shows a smooth, epithelialized corneal surface with minimal scarring and inflammation (C and F).

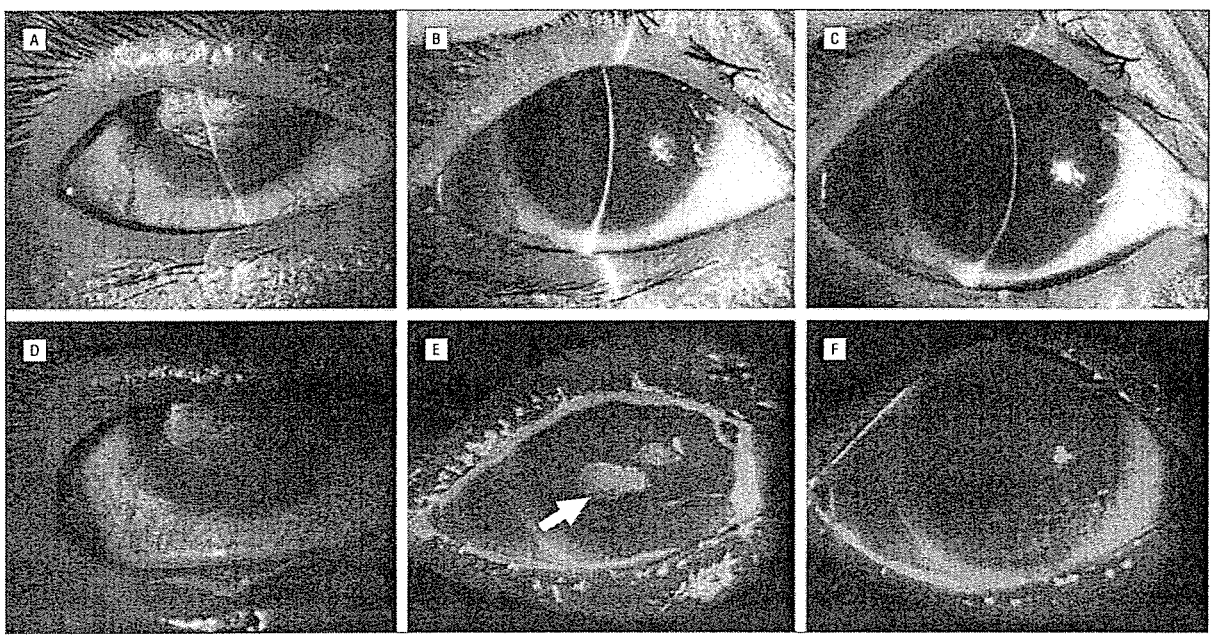

Figure 5. External appearance (A-C) with corresponding fluorescein staining (D-F) of a patient with Stevens-Johnson syndrome who underwent cultivated epithelial transplantation (patient 6). Preoperative appearance demonstrates severe keratinization of the cornea with superior symblepharon formation (A and D). A small corneal epithelial defect was noted 3 months after surgery. The arrow indicates the epithelial defect stained with fluorescein (B and E). The epithelial defect healed over from the adjacent oral epithelium, and the corneal surface was noted to be smooth and epithelialized, with minimal scarring and inflammation (C and F).

were too low to be detected in subjects with SJS, other subjects with severe OSD, and healthy controls, suggesting that the serum sFasL level would not be an impediment in our culture system. To our knowledge, our recent study is the first to evaluate the suitability of AS from patients with SJS in supporting in vitro epithelial cell proliferation.[32] We demonstrated that oral epithelial cells cultured in AS-supplemented medium had similar prolif-

erative capacities compared with FBS-supplemented cultures, which ensures that the regenerative potential of these cells was similarly maintained in both culture systems.[32]

Most of the previous studies on bioengineered corneal epithelial equivalents for clinical transplantation have relied primarily on FBS-supplemented medium in the culture process.[11-20] In this study, we demonstrated that the morphological appearance of AS-derived cultivated oral epithelium was similar to that of normal in vivo cornea and FBS-derived cultures. Immunohistochemical analysis confirmed the presence of the keratin 4–keratin 13 pair, which is consistent with that of nonkeratinized, stratified epithelia. The cultivated oral epithelial cells also demonstrated positive staining for keratin 3, a marker for corneal differentiation,[33] suggesting that these epithelial sheets bore some similarities to normal corneal epithelium. The AS-derived oral epithelial equivalents also demonstrated the presence of basement membrane–related proteins and hemidesmosomes (integrins α6 and β4),[34] which are important for ensuring graft integrity during surgical manipulation and after transplantation. The cultivated oral epithelial sheets demonstrated good cell-to-substrate adhesion, and graft integrity was maintained throughout the follow-up.

The ability of transplanted oral epithelial equivalents to continue to regenerate and replenish the corneal epithelial surface is of critical importance when evaluating their use for clinical transplantation. We demonstrated that AS-derived cultivated oral epithelial transplantation achieved complete corneal epithelialization within 2 to 5 days, which is similar to our previous results with cultivated oral epithelial transplantation using FBS-supplemented culture medium.[18,19] The corneal surface of all eyes remained clear and smooth and was covered with transplanted epithelium at the last follow-up visit, with the longest follow-up being 19 months. Although this was a noncomparative clinical study, the clinical results of transplanting AS- and FBS-derived cultivated oral epithelial equivalents were similar to those of our previous clinical experience,[19] suggesting that AS-cultivated epithelial transplantation is a safe and effective procedure for the treatment of severe OSD.

CONCLUSIONS

We have demonstrated the effective use of AS-derived cultivated autologous oral epithelial transplantation for the treatment of severe limbal stem cell deficiency. This novel treatment modality has important clinical implications because it eliminates the use of bovine material in the culture process, reduces the risk of allograft rejection and transmission of infection, and reduces the need for long-term corticosteroid and immunosuppressive therapy. This study has brought us one step closer toward developing safer xenobiotic-free autologous bioengineered products that are derived entirely from the patient's own tissue. The successful use of completely autologous bioengineered tissue equivalents for clinical transplantation represents a significant advancement in the field of ocular bioengineering and transplantation.

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