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

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

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

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

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

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

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

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

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

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

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

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

Materials and Methods

All experimental procedures and clinical applications introduced here were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

Preparation of Amniotic Membrane

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

Subjects and Harvesting of Serum

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

Cultivation of Human Corneal and Oral Epithelial Cells

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

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

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

Quantitation of Proliferative Capacity and Clonal Growth of Cells

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

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

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

The CFE was defined as follows

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

The Development of Corneal and Oral Epithelial Equivalents

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

Immunohistochemistry

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

TABLE 1. Primary Antibodies and Source

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

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

Electron Microscopy

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

RESULTS

Proliferative Capacity and Clonal Growth

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

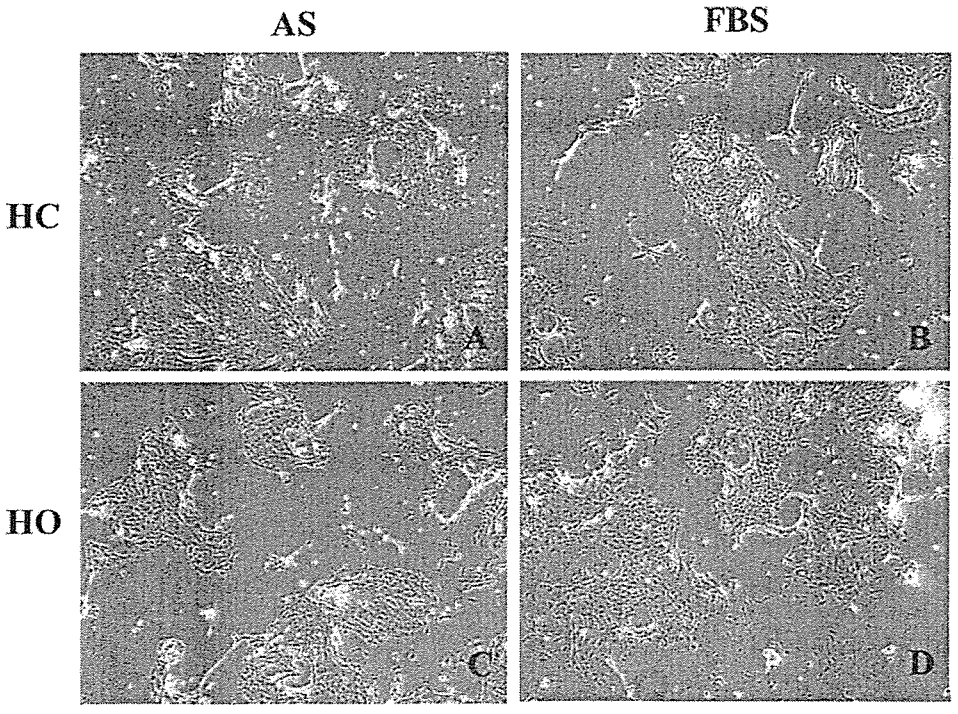

FIGURE 1. Representative phase contrast appearance of human corneal epithelial cells (A, B) and human oral epithelial cells (C, D) cultivated in AS- (A, C) and FBS-supplemented (B, D) media on day 6. In both AS- and FBS-supplemented media, human corneal and oral epithelial cells formed colonies consisting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

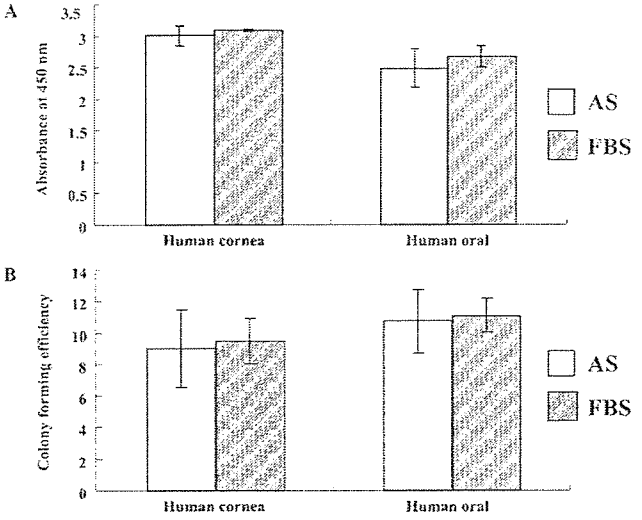

FIGURE 2. (A) BrdU ELISA cell proliferation assay of corneal and oral epithelial cells cultivated in AS- (n = 12) and FBS-supplemented (n = 12) media. The bars show the mean values of BrdU absorbance in each culture condition. Proliferation indices (PI) of human corneal epithelial cells were 3.00 ± 0.16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

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

Differentiation of Cultivated Corneal and Oral Epithelial Cells

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

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

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

Basement Membrane Assembly Protein Expression

Immunohistochemistry showed linearly positive staining of integrin $\alpha 6$ (Figs. 5A1–5A3, Figs. 6A1–6A3), integrin $\beta 4$ (Figs. 5B1–5B3, Figs. 6B1–6B3), collagen IV (Fig. 5D13, Figs. 6D1–6D3), collagen VII (Figs. 5E1–5E3, Figs. 6E1–6E3), and laminin 5 (Figs. 5F1–5F3, Figs. 6F1–6F3) on the basement membrane side of corneal and oral epithelial cells. In contrast, integrin $\beta 1$ was expressed in the cell membrane of epithelial cells (Figs. 5C1–5C3, Figs. 6C1–6C3). These AS- and FBS-derived epithelial sheets maintained the phenotypic characteristics of normal in vivo corneal and oral epithelia.

Electron Microscopy

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

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

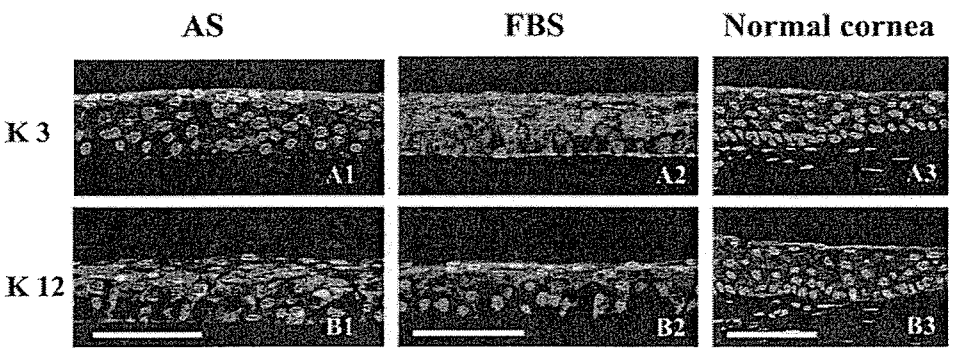

FIGURE 3. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (A1, B1) and FBS-supplemented (A2, B2) media, compared with normal in vivo cornea epithelium (A3, B3). In all 3 epithelia, cornea-specific keratins 3 (A1–A3) and 12 (B1–B3) were expressed in the superficial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

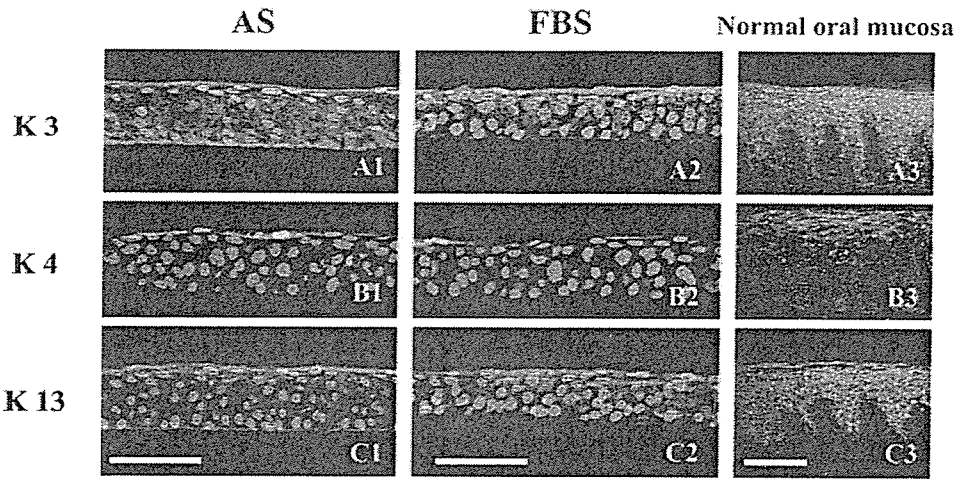

FIGURE 4. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (A1–C1) and FBS-supplemented (A2–C2) media, compared with normal in vivo oral epithelium (A3–C3). In normal oral epithelium, keratin 3 (A3) and keratin 13 (C3) were expressed in all epithelial layers, except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

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

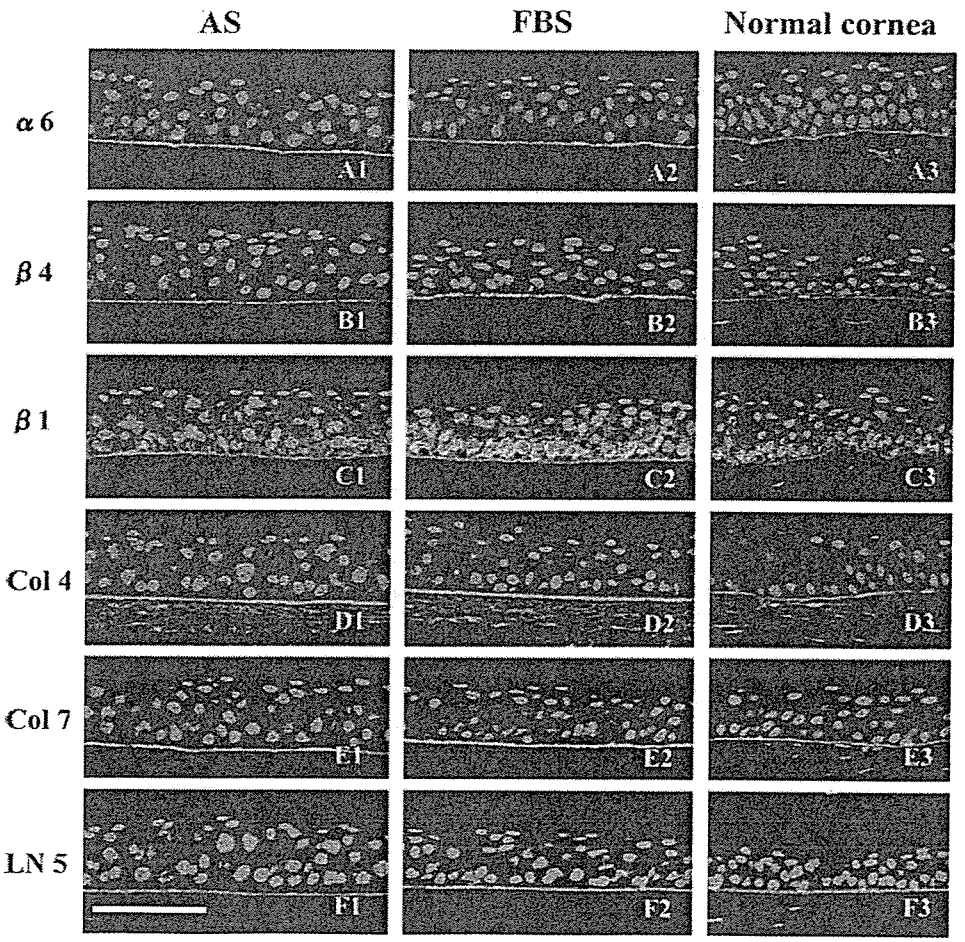

FIGURE 5. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (A1–F1) and FBS-supplemented (A2–F2) media, compared with normal in vivo cornea epithelium (A3–F3). There was linear positive staining of integrin α6 (A1–A3), integrin β4 (B1–B3), collagen IV (D1–D3), collagen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

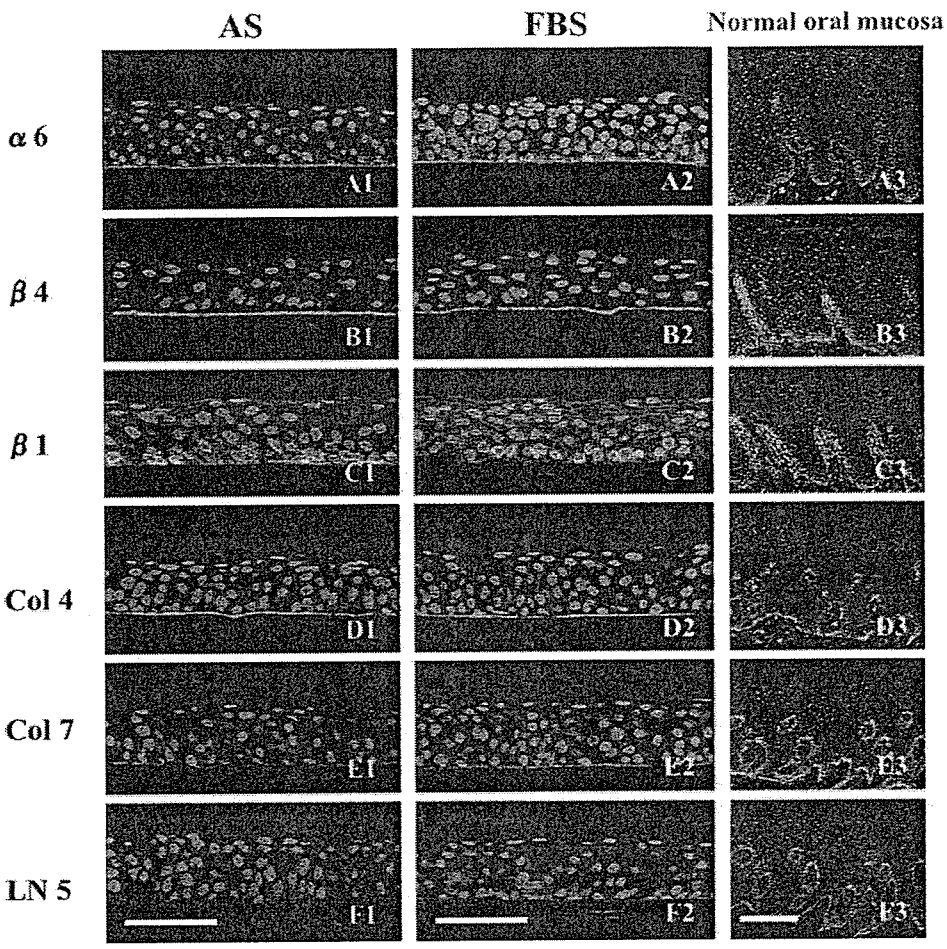

FIGURE 6. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (A1–F1) and FBS-supplemented (A2–F2) media, compared with normal in vivo oral epithelium (A3–F3). There was linear positive staining of integrin α6 (A1–A3), integrin β4 (B1–B3), collagen IV (D1–D3), collagen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

DISCUSSION

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

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

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

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

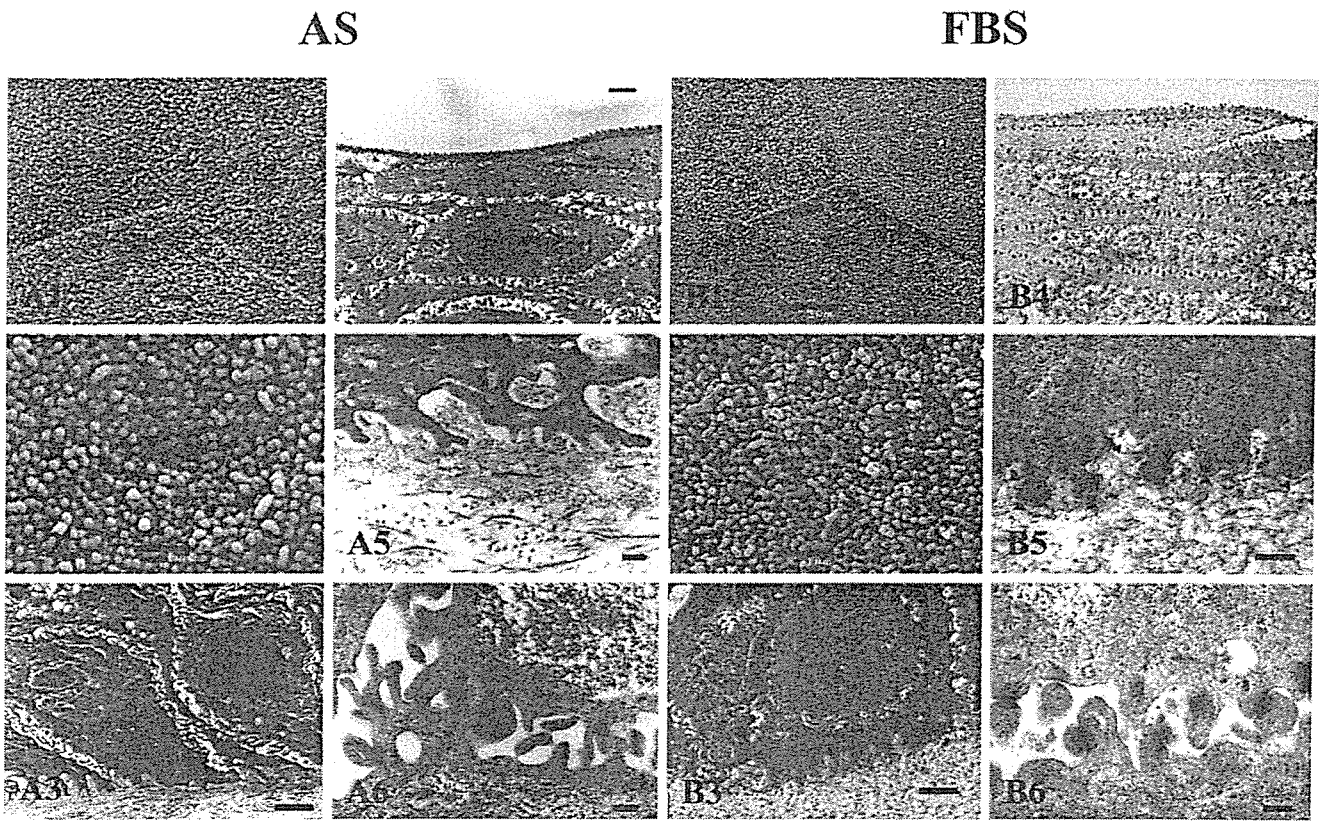

FIGURE 7. Scanning electron microscopic examination showed a continuous layer of flat squamous polygonal epithelial cells in the cultivated corneal epithelial cells when using AS (A1) and FBS (B1). These cells were closely attached to each other with tightly opposed cell junctions and distinct cell boundaries.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

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

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

Acknowledgments

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

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Novel clinical application of sterilized, freeze-dried amniotic membrane to treat patients with pterygium

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ABSTRACT.

Purpose: **To evaluate the use of sterilized, freeze-dried amniotic membrane (FD-AM) transplantation for pterygium surgery.**

Methods: **This study involved a prospective, non-comparative, interventional case series. Thirteen eyes of 13 patients with primary (eight eyes) or recurrent (five eyes) pterygium were studied. After excision of the pterygium fibrous tissues and application of intraoperative use of mitomycin-C, sterilized FD-AM was sutured over the bare scleral defect. The integrity of the FD-AM graft, epithelialization over the FD-AM, pterygium recurrence and postoperative complications were evaluated.**

Results: **Postoperatively, the FD-AM was well retained in all patients, and complete epithelialization over the transplanted membrane was achieved within 1–2 weeks. All patients demonstrated early resolution of ocular inflammation and there was no recurrence of pterygium in any of the treated patients during the mean follow-up of 13.9 ± 6.0 months. No ocular complications were noted following transplantation.**

Conclusion: **Sterilized FD-AM showed excellent biocompatibility on the human ocular surface. This novel and promising biomaterial may be a useful alternative to conjunctival grafting in the treatment of pterygium.**

Key words: amniotic membrane – freeze-dry – pterygium – biomaterial – ocular surface

Acta Ophthalmol. Scand. 2006: 84: 401–405

doi: 10.1111/j.1600-0420.2006.00667.x

Introduction

The fresh human amniotic membrane (AM), the innermost layer of the placental membrane, is a thin, semitransparent tissue comprised of a single layer of ectodermally derived columnar cells, a thick, continuous basement membrane, and subjacent avascular stroma (van Herendael et al. 1978). Cryopreserved AM has been shown to possess anti-inflammatory (Kim et al. 2000; Solomon et al. 2001a), antifibroblastic activity (Tseng et al. 1999) and antiangiogenic properties, as well having limited immunogenicity (Akle et al. 1981; Hao et al. 2000). Because of these desirable characteristics, cryopreserved AM has been used in a wide variety of ocular surface reconstructive procedures.

Pterygium is a common external ocular disease characterized by fibrovascular overgrowth of degenerative conjunctiva onto the cornea, together with associated ocular inflammation. A wide variety of surgical methods have been employed in its treatment, with the aims of ensuring a good cosmetic result, reducing the risk of recurrence, and minimizing complications. These include bare sclera excision, with or without the adjunctive use of mitomycin-C or, β-irradiation (Manning et al. 1997; Lam et al. 1998), or wound closure using a conjunctival autograft (Lewallen 1989), conjunctival limbal autograft (Mutlu et al. 1999), cryopreserved human amniotic membrane (Prabhasawat et al. 1997; Solomon et al. 2001b), or more recently, autologous cultivated conjunctival transplantation (Ang et al. 2005). Amniotic membrane transplantation, used alone, or in combination with conjunctival autograft, limbal autograft or mitomycin-C (MMC) has been shown to be an effective method for treating pterygium, although its reported clinical efficacy has varied between reports (Prabhasawat et al. 1997; Shimazaki

et al. 1998; Ma et al. 2000; Solomon et al. 2001b; Shimazaki et al. 2003).

The anti-inflammatory, antifibrotic and antiangiogenic properties of cryopreserved AM, as well as its ability to promote epithelialization and maintain the epithelial stem cell niche make it useful in the treatment of a variety of ocular surface diseases, including pterygium. However, safety and logistical issues pose potential problems with its use. Preparation of AMs for clinical use usually entails several washings with buffered saline containing antibiotics. However, complete disinfection is not always guaranteed. Human AMs, being biological tissues, pose the potential risk of transmission of infection from the donor, or from contamination or inadequate disinfection during its processing and preservation (Adds et al. 2001; von Versen-Hoynck et al. 2004). As such, proper and reliable sterilization of AM is vital to render it completely safe for clinical applications. Furthermore, cryopreservation of AM requires the use of expensive, space-consuming freezers. These are hindrances to the widespread availability and usage of AM in many parts of the world, particularly in less developed countries. In order for AM to be ideally suited for widespread clinical use, it needs to be sterile, free of contamination, easily obtainable, and its transport and prolonged storage at room temperature should not have any adverse effects on its quality.

Our group previously reported that sterilized, freeze-dried amniotic membrane (FD-AM) produced by our unique protocol retained much of the physical, biological and morphological characteristics of cryopreserved AM (Nakamura et al. 2004a). Freeze-dried amniotic membrane is wafer-thin, type 4 collagen-rich substrate. Although its flexibility upon hydration is slightly less than that of cryopreserved AM, we found it was a useful substrate for ocular surface reconstruction in a rabbit model (Nakamura et al. 2004a). In the current study we evaluated the biocompatibility of FD-AM on the human ocular surface. We successfully applied this novel, safe and convenient biomaterial to the eyes of 13 patients with pterygium. This report is the first step in assessing the feasibility of using FD-AM for pterygium surgery and ocular surface transplantation.

Materials and Methods

Subjects

The study included 13 eyes of 13 consecutive patients with pterygium who underwent FD-AM transplantation at our hospital between July 2003 and December 2004. All patients were treated by the same two surgeons (SK and TI). All patients were followed-up for more than 6 months after transplantation. The Human Studies Committee of Kyoto Prefectural University of Medicine approved the transplantation of FD-AM and we obtained prior oral and written informed consent from all patients.

Preparation of sterilized, freeze-dried AM

The FD-AM was prepared according to our previously reported method (Nakamura et al. 2004a). Briefly, after obtaining proper informed consent in accordance with the tenets of the Declaration of Helsinki for research involving human subjects, and with approval by the Institutional Review Board of Kyoto Prefectural University of Medicine, human AM was obtained from donors undergoing elective caesarean section. The AM was washed with sterile phosphate buffered saline containing antibiotics and antimycotics. Amniotic epithelial cells were removed by incubation with 0.02% ethylene diamine tetra-acetic acid. Denuded AM was freeze-dried under vacuum conditions, vacuum-packed at room temperature, and sterilized by gamma-irradiation (25 kGy). The resultant material was wafer-like, very lightweight and thin, easy to handle, and could be sutured without tearing.

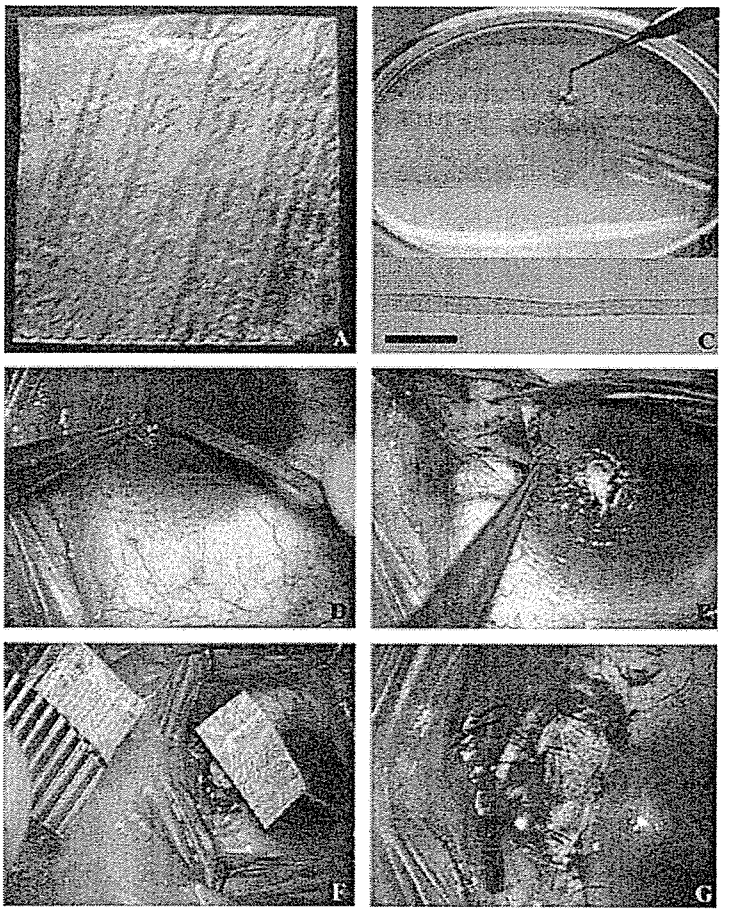

Fig. 1. (A) The sterilized, freeze-dried AM was wafer-like and thin. (B) It became smooth and relatively flexible on hydration. (C) It is a well compacted material. (Haematoxylin and eosin staining; scale bar 20 μm.) (D) In surgery, the head of the pterygium is carefully excised with forceps as bluntly as possible. (E) Subconjunctival fibrotic tissue is then excised as much as possible and the sclera is exposed. (F) FD-AM, trimmed to the appropriate size to fit the bare sclera, is then placed on the sclera. (G) The FD-AM is secured with 10–0 nylon sutures.

Table 1. Characteristics of cases and clinical outcomes.

Case	Age (years)	Gender	Diagnosis	Eye	Epithelialization at 2 weeks	Recurrence	Complications	Visual acuity		
								Preop	Postop	Follow-up (months)
1	76	M	PP	R	+	–	–	20/50	20/40	24
2	70	M	PP	R	+	–	–	20/60	20/60	20
3	58	M	PP	L	+	–	–	20/15	20/15	20
4	75	M	PP	R	+	–	–	20/25	20/25	20
5	62	M	RP	R	+	–	–	20/15	20/15	18
6	65	M	PP	R	+	–	–	20/40	20/40	16
7	55	F	PP	R	+	–	–	20/15	20/15	14
8	71	M	RP	L	+	–	–	4/100	6/100	10
9	77	F	RP	R	+	–	–	30/50	30/50	9
10	44	M	PP	R	+	–	–	20/20	20/20	9
11	67	F	RP	R	+	–	–	20/50	20/50	8
12	68	M	RP	L	+	–	–	20/20	20/20	7
13	62	F	PP	R	+	–	–	30/50	20/20	6

M = male; F = female; PP = primary pterygium; RP = recurrent pterygium.

It could be preserved at room temperature and became relatively smooth and flexible upon hydration (Fig. 1A, B). Haematoxylin and eosin staining showed it to be a compact, collagen-rich substrate (Fig. 1C).

Surgical procedures

Topical anaesthesia with 4% lignocaine was used in most patients. The head of the pterygium was first removed with blunt dissection (Fig. 1D). After extensive dissection of subconjunctival fibrotic tissue with surgical scissors to expose the sclera (Fig. 1E), the residual subconjunctival tissue was treated for 5 mins with 0.04% MMC followed by vigorous repeated washing with saline. The surrounding conjunctiva was secured onto the bare sclera with 6–0 silk sutures. Using 10–0 nylon sutures, the FD-AM was then transplanted over the bare scleral defect with the epithelial basement membrane side facing up (Fig. 1F, G). The free edge of the FD-AM was imbricated under the host conjunctiva to allow the outgrowth of conjunctival epithelium onto the FD-AM. Finally, the area of transplantation was covered with a therapeutic soft contact lens. Patients were evaluated on postoperative days 1, 3, 7, 14 and 30 and then every 3 months. The sutures were removed 2 weeks after the operation. Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone were instilled four times a day for 2–3 months; this treatment was subsequently replaced with the administration of fluorometholone three to four times a day for the next few months. Recurrence was defined as any fibrovascular growth beyond the limbus onto the cornea.

Results

Our subjects included nine men and four women; their mean age was 65.4 ± 9.33 years. Of the 13 eyes, eight had primary pterygium and five had recurrent pterygium. The mean follow-up period was 13.9 ± 6.0 months. The clinical data for the 13 patients are presented in Table 1.

The FD-AM was easy to handle and could be easily sutured. The membrane's flexibility increased following hydration. Postoperatively, all transplanted FD-AMs maintained their graft integrity and were well retained. In all 13 eyes, complete conjunctival epithelialization over the transplanted FD-AM was achieved within 1–2 weeks (Fig. 2). The surrounding conjunctival inflammation began to gradually subside and slit-lamp examination confirmed that conjunctival fibrosis was successfully suppressed in all patients. We encountered no significant intra- or postoperative complications at the transplantation site and none of the 13 patients suffered loss of visual acuity. There was no recurrence of the pterygium during the follow-up period (Fig. 3). At the last follow-up visit, all transplanted FD-AMs continued to show excellent biocompatibility with the ocular surface. All treated eyes

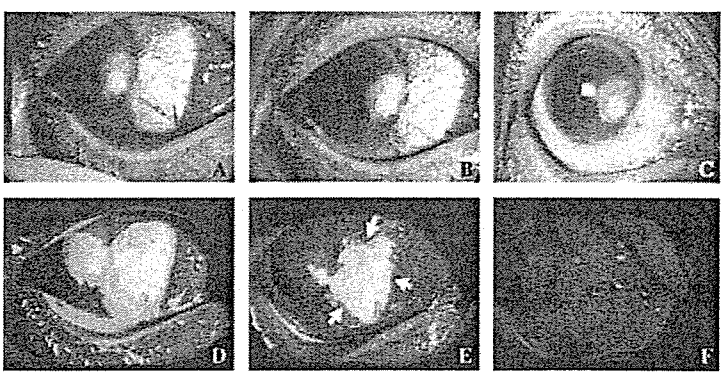

Fig. 2. Representative primary time-course slit-lamp photographs of pterygium eyes after FD-AM transplantation (case 5), taken with and without fluorescein. (A, D) At 24 hours after transplantation, the eye manifested epithelial defects at the transplanted site and host cornea. (B, E) At 72 hours post-transplantation, surrounding conjunctival epithelium gradually migrated onto the FD-AM. (C, F) At 1 week, the ocular surface including the transplanted AM was completely epithelialized and stable without defects.

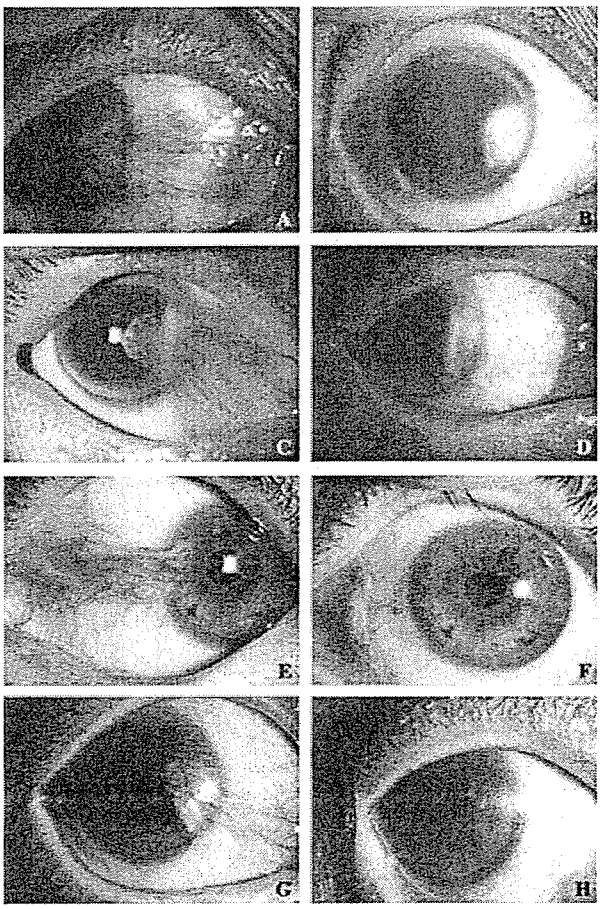

Fig. 3. Representative slit-lamp photographs taken before transplantation (A) case 1, (C) case 2, (E) case 3, (G) case 6; and at the last follow-up visit (B) case 1, (D) case 2, (F) case 3, (H) case 6. Before transplantation, the eyes manifested fibrovascular overgrowth of degenerative conjunctiva onto the cornea. At the last follow-up visit, fibrosis was markedly suppressed and the conjunctival surface was stable without inflammation or rejection.

remained stable without ocular infection or graft rejection.

Discussion

As AM possesses several beneficial characteristics such as the promotion of epithelialization and inhibition of fibrosis (Tseng et al. 1997; Meller & Tseng 1999; Lee et al. 2000), it has been successfully used in the treatment of patients with pterygium (Prabhasawat et al. 1997; Shimazaki et al. 1998; Ma et al. 2000; Shimazaki et al. 2003). Complete sterilization of the AM is of utmost importance to render it safe for clinical use. In the evaluation of FD-AM as an effective biomaterial, its biocompatibility on the human ocular surface must first be established. We previously reported that transplanted FD-AM adapted well in the corneal stroma and bare sclera of rabbits and that there was no evidence of subepithelial cell infiltration, stromal oedema, neovascularization, inflammation, or rejection (Nakamura et al. 2004a). All transplanted FD-AMs in our current clinical study also adapted well to the bare host sclera without dissolution following pterygium excision. There was no immunological rejection of the transplanted FD-AM in any of our 13 patients. These results indicate that the FD-AM we produced exhibited excellent biocompatibility with the human ocular surface and suggests that FD-AM may be used for the treatment of pterygium.

Conjunctival autografting is safe and effective. However, the procedure is more time-consuming and technically demanding to perform compared with other procedures, such as bare sclera excision. We must also point out that a wide variation in recurrence rates exists because of the differences in surgical procedure and surgeons' experience. As the superior bulbar conjunctiva is the preferred site for autograft harvesting, subsequent glaucoma surgery in these patients is associated with a high risk of failure. As such, the use of FD-AM may therefore be a useful alternative to conjunctival autograft surgery as it eliminates the need for harvesting autografts and causing iatrogenic injury to the donor site, which is particularly important when dealing with more extensive pterygium. Further evaluation of the relative efficacy of conjunctival autografts and FD-AMs, with or without adjunctive MMC, would therefore provide valuable information regarding pterygium management.

The coverage of a large area by the AM following the excision of pterygium may promote the proliferation and differentiation of the remaining conjunctival epithelium. This may have an inhibitory effect on postoperative inflammation and may reduce the risk of complications such as postoperative infections and scleral melting. The suppression of postoperative inflammation is essential for achieving successful results following pterygium surgery (Prabhasawat et al. 1997; Shimazaki et al. 1998). Complete epithelialization over the FD-AM was achieved within 2 weeks, which was similar to our previous experience with cryopreserved AM, suggesting that FD-AM may be a useful substrate for epithelium migration and proliferation. Furthermore, the large size of AM allows the extensive excision of fibrotic scar tissue. These attributes may contribute to favourable clinical results in the treatment of pterygium. In addition, from a cosmetic point of view, the outcome of this transplantation procedure was highly satisfactory.

Also important for the achievement of satisfactory clinical results in patients treated for pterygium is the suppression of postoperative fibrosis. Amniotic membrane exerts antiscarring effects via the transforming growth factor-β pathway (Tseng et al. 1999). Although FD-AM may lose several characteristics during the freeze-drying process, FD-AM combined with MMC treatment strongly suppressed postoperative fibrosis in the transplantation area. In our current study, we

were unable to determine the exact efficacy of FD-AM in prevention of inflammation and pterygium recurrence. Treatment with MMC alone is effective in reducing pterygium recurrence and postoperative topical steroids would also reduce postoperative inflammation and scarring. In view of these findings, further studies comparing the use of FD-AM alone, FD-AM with MMC and cryopreserved AM, with or without MMC, are needed to clarify these points.

We found that, in our patients, the transplanted area had minimal inflammation and scarring, suggesting that our procedures can be used in patients with severe cicatricial diseases requiring conjunctival reconstruction, such as Stevens–Johnson syndrome or chemical injury. We are in the process of adapting our technique to treat patients with ocular surface disease with severe symblepharon that requires fornix reconstruction. In addition, we are also using this biomaterial as a substrate for cultivated corneal and oral epithelial sheets for ocular surface reconstruction (Koizumi et al. 2001; Nakamura et al. 2004b).

In conclusion, ours is the first demonstration of the excellent biocompatibility of FD-AM for clinical use. Freeze-dried AM offers important advantages over cryopreserved AM because it is free of contamination, may be easily transported and stored, and can be used readily with minimal preparation at the time of surgery. Although the longterm and comparative clinical results of these grafts await confirmation, this modality of treatment may be a useful alternative to conjunctival graft or cryopreserved AM surgery for the treatment of pterygium.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labour and Welfare (H16-Saisei-007) and the Japanese Ministry of Education, Culture, Sports, Science and Technology 14657450, a research grant from the Kyoto Foundation for the Promotion of Medical Science, and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

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Received on June 8th, 2005.
Accepted on December 21st, 2005.

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Transplantation of Autologous Serum-Derived Cultivated Corneal Epithelial Equivalents for the Treatment of Severe Ocular Surface Disease

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Purpose: To evaluate the use of autologous serum (AS)-derived cultivated corneal epithelial transplantation for the treatment of severe ocular surface disease.

Design: Retrospective noncomparative case series.

Participants: Nine eyes from 9 patients with total limbal stem cell deficiency were studied. These consisted of 2 eyes with Stevens–Johnson syndrome, 1 with chemical injury, 1 with ocular cicatricial pemphigoid, 1 with Salzmann corneal dystrophy, 1 with aniridia, 1 with graft-versus-host disease, and 2 with idiopathic ocular surface disease.

Methods: Autologous serum obtained from patients was used for cultivating corneal epithelial cells on an amniotic membrane substrate. These AS-derived corneal epithelial equivalents were compared with those derived from fetal bovine serum (FBS)-supplemented medium. At the time of surgery, complete removal of the corneal pannus and conjunctiva up to 3 mm from the limbus was performed. Allogeneic (7 cases) and autologous (2 cases) AS-derived cultivated corneal epithelial equivalents were transplanted onto the ocular surface. Postoperative follow-up included serial slit-lamp examinations with fluorescein staining, as well as photographic documentation.

Main Outcome Measures: Ocular surface reconstruction with corneal epithelialization, graft integrity, visual acuity, and postoperative complications.

Results: The corneal epithelial sheets cultivated in AS- and FBS-supplemented media were morphologically similar, and demonstrated the normal expression of tissue-specific keratins and junctional specialization assembly proteins. After transplantation, complete corneal epithelialization was achieved within 2 to 5 days. All eyes demonstrated an improvement in visual acuity by ≥2 lines. During the follow-up period of 14.6±4.36 months, the corneal surface of all patients remained stable and transparent, without significant complications.

Conclusions: Transplantation of AS-derived cultivated corneal epithelial equivalents was shown to be a feasible method of treating patients with severe ocular surface disease. The use of AS is of clinical importance in the development of autologous xenobiotic-free bioengineered ocular surface equivalents for clinical transplantation. *Ophthalmology 2006;113:1765–1772 © 2006 by the American Academy of Ophthalmology.*

Severe ocular surface disease, such as Stevens–Johnson syndrome, ocular cicatricial pemphigoid and, chemical injury, are devastating conditions that represent a major clinical challenge. In such cases, the corneal epithelial stem cells in the corneal limbus are destroyed, and invasion of the corneal surface by the neighboring conjunctival epithelium results in neovascularization, chronic inflammation, ingrowth of fibrous tissue, and stromal scarring.[1–3] Patients with these conditions do poorly with conventional corneal transplantation. Ocular surface reconstruction and corneal epithelial replacement by means of keratoepithelioplasty and limbal transplantation, often combined with amniotic

Originally received: October 12, 2005.
Accepted: April 24, 2006. Manuscript no. 2005-982.

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Supported in part by grants-in-aid for scientific research from the Japanese Ministry of Health, Labor, and Welfare, Tokyo, Japan (no.: H16-Saisei-007), and Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan (Kobe Translational Research Cluster); a research grant from the Kyoto Foundation for the Promotion of Medical Science, Kyoto, Japan; and the Intramural Research Fund of Kyoto Prefectural University of Medicine.

The authors have no financial interest in this work.

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ISSN 0161-6420/06/$–see front matter
doi:10.1016/j.ophtha.2006.04.030

Table 1. Characteristics of Cases

Age/Gender	Disease	Eye	Donor	Days to Reach Confluence	Quality of Culture Sheet	Previous Surgery
33/F	SJS	Right	Allo	6	Excellent	–
27/M	SJS	Left	Allo	7	Excellent	COET
81/F	Idiopathic	Right	Allo	8	Good	–
49/M	Chemical	Right	Auto	6	Excellent	–
70/M	Idiopathic	Right	Auto	9	Excellent	Ptosis
42/F	Salzmann	Left	Allo	6	Excellent	–
71/M	OCP	Left	Allo	8	Excellent	–
41/F	Aniridia	Right	Allo	7	Excellent	–
47/M	GVHD	Right	Allo	7	Excellent	–

ALKP = automated lamellar keratoplasty; Allo = allologenic cultivated corneal epithelial transplantation; AMT = amniotic membrane transplantation; defect; F = female; GVHD = graft-versus-host disease; HM = hand motion; IOL = intraocular lens; M = male; OCP = ocular cicatricial pemphigoid;

membrane (AM) transplantation, have been effectively used to treat these severely damaged eyes.[4–8]

More recently, cultivated corneal epithelial stem cell transplantation has been shown to be a promising treatment modality in the management of severe limbal stem cell deficiency.[9–15] The use of fetal bovine serum (FBS)-supplemented media remains the media of choice for the ex vivo expansion of cells in corneal epithelial equivalents. The use of bovine material for cultivating epithelial cells for clinical use is an important health and safety issue; its use is associated with the risk of transmission of zoonotic infection (e.g., bovine spongiform encephalitis) and other unknown pathogens. Because bovine spongiform encephalitis cannot be detected by any known in vitro assay, the use of FBS in the culture medium is a major concern worldwide. The use of human autologous serum (AS) as an alternative to FBS is therefore significantly advantageous because it eliminates the need for bovine material in the culture process and reduces the risk of transmission of disease.

We previously showed that human serum was able to support the in vitro and in vivo proliferation of epithelial cells.[16] In our study, we evaluated the use of AS in developing cultivated corneal epithelial equivalents and compared it with conventional FBS supplemented cultures. These AS-derived cultivated epithelial equivalents were used in the treatment of patients with severe limbal stem cell deficiency. To our knowledge, a study of this nature has not been previously reported. This study has important clinical implications and provides the basis for developing safer autologous bioengineered tissues for clinical transplantation.

Materials and Methods

Subjects

All experimental procedures and clinical applications introduced here were approved by the Institutional Review Board for Human Studies of Kyoto Prefectural University of Medicine; prior informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki for research involving human subjects.

The study included 9 eyes from 9 consecutive patients with severe ocular surface disease that underwent ocular surface reconstruction with AS-derived cultivated corneal epithelial equivalents between February 2004 and April 2005. Seven eyes underwent allogeneic transplantation and 2 eyes underwent autologous transplantation. There 5 male and 4 female patients; their ages ranged from 27 to 81 years (mean 51.2±18.6 years). All were diagnosed as totally stem cell–deficient on the basis of complete disappearance of the palisade of Vogt and 360 degrees of conjunctivalization. The preoperative diagnosis was Stevens–Johnson syndrome (n = 2), ocular cicatricial pemphigoid (n = 1), chemical injury (n = 1), Salzmann corneal dystrophy (n = 1), aniridia (n = 1), graft-versus-host disease (n = 1), and idiopathic ocular surface disorder of unknown etiology (n = 2). Preoperatively, all 9 eyes manifested severe destruction of the ocular surface with total limbal stem cell deficiency. Tear production was diminished but not absent in all patients, as evidenced by the presence of a tear meniscus level with diminished tear film breakup time. All patients were followed for a minimum of 6 months.

Cultivation of Corneal Epithelial Sheet

Harvesting and Preparation of Autologous Serum. Under aseptic conditions, AS was obtained from each patient by venesection at the antecubital fossa. Thirty milliliters of blood were collected into a sterile container, centrifuged, filtered, and the resultant serum (approximately 10 ml) was purified. Each patient's serum was stored in sterile tubes at −30° C before use.

Preparation of Cultivated Corneal Epithelial Equivalent. We cultured human corneal epithelial cells using a previously reported system.[12,17–21] For allogeneic corneal epithelial cultures, human corneal limbal tissues from Northwest Lion Eye Bank (Seattle, WA) were incubated at 37° C for 1 hour with 1.2 IU neutral protease (Dispase, Basel, Switzerland) to separate the epithelial cells. The resultant corneal epithelial cells (1×10^5 cells/well) were then seeded onto denuded AM spread on the bottom of culture inserts, and cocultured with mitomycin C–inactivated 3T3 fibroblasts (2×10^4 cells/cm^2). For autologous corneal epithelial cultures, a corneal limbal epithelial explant from a 3-mm^2 biopsy of the uninjured eye was placed directly on a denuded AM carrier. The culture medium consisted of a proprietary defined keratinocyte growth medium (kindly provided by ArBlast Co. Ltd., Kobe, Japan), which included several growth factors and hormones, without any animal-derived material, supplemented with 5% AS. The cultured cells were submerged in medium for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 1 to 2 days. Cultures were incubated at 37° C in a 5% CO_2–95% air incubator, with medium change carried out every day. The AS-derived corneal epithelial equivalents were compared with corre-

Combined Surgery	Epithelialization (2–5 days)	Visual Acuity		Complication	Follow-up (mos)
		Before	*After*		
AMT	+	20/100	20/32		20
–	+	HM	20/320	ED	19
AMT	+	HM	20/100		18
–	+	CF	20/125		16
–	+	CF	20/630		14
–	+	20/2000	20/125	ED	14
PEA + IOL	+	20/63	20/25		13
ALKP + PEA + IOL	+	CF	20/200		11
AMT + PEA + IOL	+	HM	20/32		6

Auto = autologous cultivated corneal epithelial transplantation; CF = count finger; COET = cultivated oral epithelial transplantation; ED = epithelial PEA = phacoemulsification and aspiration; SJS = Stevens–Johnson syndrome.

sponding corneal epithelial equivalents prepared in FBS-supplemented medium.

Morphologic Examination of Cornea Epithelial Equivalents

The morphologic examination of the cultivated epithelial equivalents was performed on additional epithelial sheets that were cultivated in AS- and FBS-supplemented media. Based on the histologic appearance of the culture sheet, we graded the quality of the cultivated epithelial sheet as follows: excellent = entire sheet demonstrated normal cell differentiation and stratification, maintenance of the cuboidal basal layer, and good adhesion to the underlying AM substrate; good = >80% of the epithelial sheet demonstrated normal cell differentiation and stratification, with some areas that were less adherent; or average = between 50% and 80% of the epithelial sheet demonstrated normal cell differentiation and stratification, with basal cells adopting a more columnar or flattened appearance, and areas that were detached from the AM substrate.

Cryostat sections (7 μm thick) were placed on gelatin-coated slides, air dried, then rehydrated in phosphate-buffered saline at room temperature for 15 minutes. Morphologic analysis was performed by staining with hematoxylin and eosin. Immunohistochemical studies of cell junction specialization–related proteins and tissue-specific keratins in corneal epithelial sheets cultivated using AS or FBS were carried out after our previously described method.[21,22] To block nonspecific binding, the tissues were incubated with 2% bovine serum albumin at room temperature for 30 minutes. Subsequently, the sections were incubated at room temperature for 1 hour with primary antibodies to keratin 12 (Transgenic, Kumamoto, Japan), ZO-1 (Zymed, San Francisco, CA), desmoplakin (Progen, Heidelberg, Germany), integrin α6 (Chemicon, Temecula, CA), and collagen 7 (Chemicon). Control incubations were with the appropriate normal mouse and rabbit IgG (Dako, Kyoto, Japan) at the same concentration as the primary antibody, and omission of the primary antibody for the respective specimen. After staining with the primary antibody, the sections were incubated at room temperature for 1 hour with appropriate secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The sections were coverslipped using antifading mounting medium containing propidium iodide (Vectashield; Vector, Burlingame, CA) and examined by confocal microscopy (Olympus Fluoview, Tokyo, Japan).

Surgical Procedure

We performed a 360-degree conjunctival peritomy 3 mm from the limbus, and removed all perilimbal scarred or inflamed subconjunctival tissue down to 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 C for 5 minutes, followed by vigorous repeated washing with saline.[12] The cultivated corneal epithelial sheet was placed over the corneal surface and secured in place with 10-0 nylon sutures at the limbus. The integrity of the cultivated epithelium was confirmed by fluorescein staining at the end of surgery, and the ocular surface was protected with a medical-use bandage contact lens.

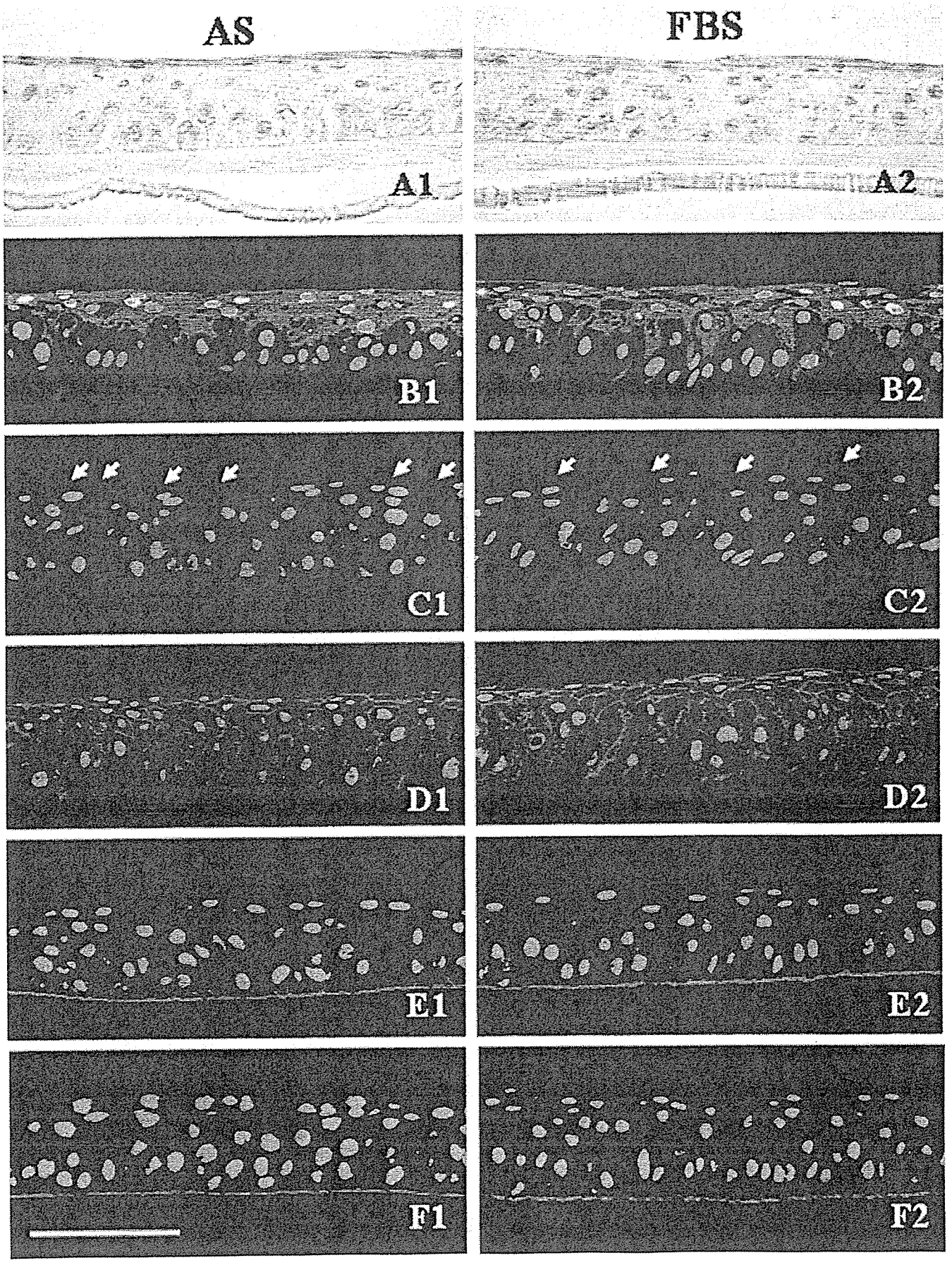
Postoperatively, 0.3% ofloxacin and 0.1% dexamethasone eye drops were instilled 4 times a day. The doses were tapered to a maintenance dose of 2 to 3 times a day after 2 to 3 months, depending on the severity of inflammation. For allogeneic transplantation, systemic betamethasone (1 mg/day), cyclophosphamide (50 mg/day), and cyclosporine (100 mg/day) were administered orally to reduce postoperative inflammation, scarring, and allograft rejection. Renal and liver functions were monitored periodically. Cyclophosphamide and cyclosporine were discontinued between 1 and 3 months after surgery.

Results

Morphology of Cultivated Corneal Epithelial Equivalent

The baseline data regarding the corneal epithelial culture are summarized in Table 1. Within 5 to 8 days in culture, a confluent sheet of corneal epithelial cells was established on the AM substrate. After 2 weeks, the cultivated corneal epithelium consisted of 4 to 6 cell layers and was composed of a well-conserved basal layer formed by cuboidal cells and several suprabasal wing and flat layers. In 8 of 9 instances, the quality of the cultivated epithelial sheets was excellent. Hematoxylin and eosin staining showed that the histology of corneal epithelial cells cultivated in AS- and FBS-supplemented media were comparatively similar (Fig 1A1, A2).

The expression patterns of tissue-specific keratins and cell junctional specialization-related proteins in cultivated corneal epithelium were similar between AS- and FBS-derived corneal epithelial equivalents. In the AS-cultivated corneal epithelial sheet, the cornea-specific keratin 12 was expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal cell layers (Fig 1B1, B2). ZO-1, which is a tight junction-related component, was expressed in the apical surface of cultivated epithelial sheet (Fig 1C1, C2). Desmoplakin, which is a cell–cell junction component, was expressed in the cell membrane of epithelial cells (Fig 1D1, D2). The basement mem-


AS
FBS
A1
A2
B1
B2
C1
C2
D1
D2
E1
E2
F1
F2

brane assembly proteins, integrin α6 and collagen 7, showed linear positive staining on the basement membrane side of basal cells (Fig 1E1–F2).

Clinical Results

The clinical data and surgical outcomes of the 9 patients are also summarized in Table 1. Before transplantation, all eyes manifested severe destruction of the ocular surface, with severe limbal stem cell deficiency (Fig 2, left column). Two to 5 days after transplantation, the corneal surface in all treated eyes was clear and smooth, and fluorescein staining confirmed that the entire corneal surface was covered by the cultivated corneal epithelium (Fig 2, middle column). The transplanted epithelial sheet was surrounded 360 degrees by conjunctival epithelial defects, indicating no contamination of the host conjunctival epithelium. Within a short time after surgery, the conjunctival inflammation was found to have rapidly subsided in all patients.

Patients were followed for 14.6±4.4 months, with the longest follow-up being 20 months. Successful engraftment was achieved in all patients with none of the grafts sloughing off. Slit-lamp examination showed that conjunctival fibrosis was successfully suppressed in all patients, with no conjunctival invasion onto the corneal surface throughout the follow-up period (Fig 2, right column). Visual acuity improved in all eyes and all were restored to good vision (postoperative visual acuity improved by ≥2 lines) at the last follow-up visit. During the follow-up period, 2 patients (cases 2 and 6) developed a small paracentral and peripheral epithelial defect, measuring approximately 2×2 mm, with mild cellular infiltration at the edge and base of the defect. This was treated as a low-grade bacterial infection with topical antibiotic medication (ofloxacin and cefmenoxime eye drops), and the epithelial defect and infiltrate gradually resolved within 2 to 3 weeks.

At the last follow-up visit, the ocular surface of the eyes with surviving transplanted cultivated epithelium remained stable without any epithelial defects. There were no significant postoperative complications and none of the patients developed graft rejection.

Discussion

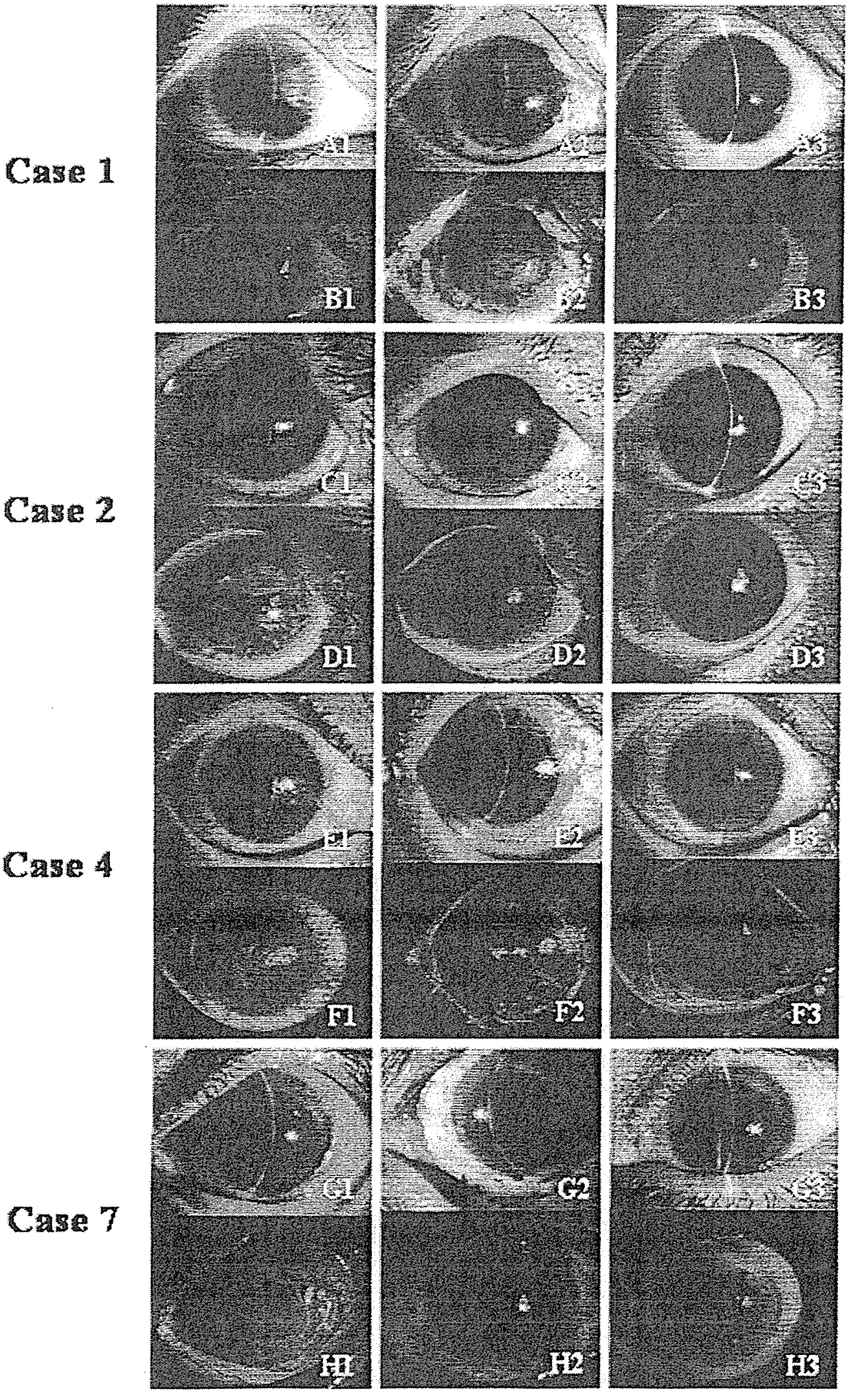
Cultivated corneal epithelial transplantation has been shown to be effective in the treatment of severe ocular surface disease. Successful epithelial cell propagation, using FBS-supplemented medium, was first described by Rheinwald and Green.[23] Since then, the use of FBS-supplemented media remains the most widely used method for cultivating ocular surface epithelial cells.[9–14,24,25] In establishing tissue equivalents for transplantation, the ideal culture condition is one that is safe from disease transmission, as well as being able to recapitulate the tissue of origin. We evaluated the biological and clinical aspects of these AS-derived corneal epithelial equivalents, and showed that they were comparable to current established methods using FBS-supplemented cultures. We describe for the first time the effective use of AS-derived cultivated corneal epithelial transplantation for the treatment of severe ocular surface disease. The novel approach of using AS as an alternative to FBS in the culture process represents a significant advancement in the development of safer bioengineered ocular surface tissues for clinical use.

A critical issue in the derivation of tissue equivalents is the ability to recapitulate a differentiated epithelial equivalent bearing the morphologic characteristics of the original tissue. Previous investigators have almost exclusively relied on FBS-supplemented medium for culturing corneal epithelial cells and developing corneal epithelial equivalents that may be used for clinical transplantation.[9–14,24,25] It is particularly important for AS-derived corneal epithelial equivalents to be able to achieve comparable results to that of FBS-derived tissues. In our study, we demonstrated that the histologic appearance of AS-derived cultivated corneal epithelium was similar to that of normal in vivo cornea as well as FBS-derived cultures in terms of cellular morphology, degree of stratification, number of cell layers, and the presence of a cuboidal basal cell layer. Immunohistochemical analyses also demonstrated the normal expression pattern of the differentiation-related proteins.

The ocular surface is exposed to significant shearing stresses from blinking and eye movement. Maintaining graft integrity is a critical factor for ensuring successful engraftment after transplantation. The AS-derived corneal epithelial equivalents not only demonstrated the presence of cell-to-cell junction–related proteins (ZO1, desmoplakin), but also the formation of basement membrane–related proteins (integrin α6 and collagen 7). These are important for ensuring graft integrity during surgical manipulation and after transplantation. These findings were similar to FBS-supplemented cultures, confirming that AS supported normal epithelial cell proliferation and differentiation and could be used safely and effectively in the development of corneal epithelial equivalents. In our study, the cultivated corneal epithelial sheet demonstrated good cell-to-substrate adhesion; successful engraftment was achieved in all patients.

The ability of transplanted corneal equivalents to continue to regenerate and replenish the corneal epithelial surface is of utmost importance when evaluating its use for clinical transplantation. Our previous experience with cultivated corneal epithelial transplantation has confirmed the long-term efficacy of this procedure for the treatment of severe ocular surface disease.[12,17–20] These cultivated corneal epithelial sheets were obtained with FBS-supplemented cultures. After transplantation, complete epithelialization

Figure 1. Representative light micrographs showed that the cultivated corneal epithelial sheet using autologous serum (AS)– and fetal bovine serum (FBS)–supplemented media had 4 to 6 layers of stratified, well-differentiated cells and the histology of these sheets were comparatively similar (A1, A2). Representative immunohistochemical results showed that cornea-specific keratins 12 (B1, B2) were expressed in the superficial and intermediate layers, with less discernible immunostaining in the basal cell layers. Cell-to-cell junctional assemblies, such as ZO1 (C1, C2) and desmoplakin (D1, D2), were expressed at the apical surface and cell membrane of cultivated corneal epithelia sheets, respectively. We observed the linearly positive staining of integrin α6 (E1, E2) and collagen 7 (F1, F2) on the basement membrane side of corneal epithelial cells. Expression pattern of these proteins were similar between cultivated epithelial sheet using AS- and FBS-supplemented culture system. Scale bar, 100 μm.


Case 1
Case 2
Case 4
Case 7
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
F1
F2
F3
G1
G2
G3
H1
H2
H3

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**).