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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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.

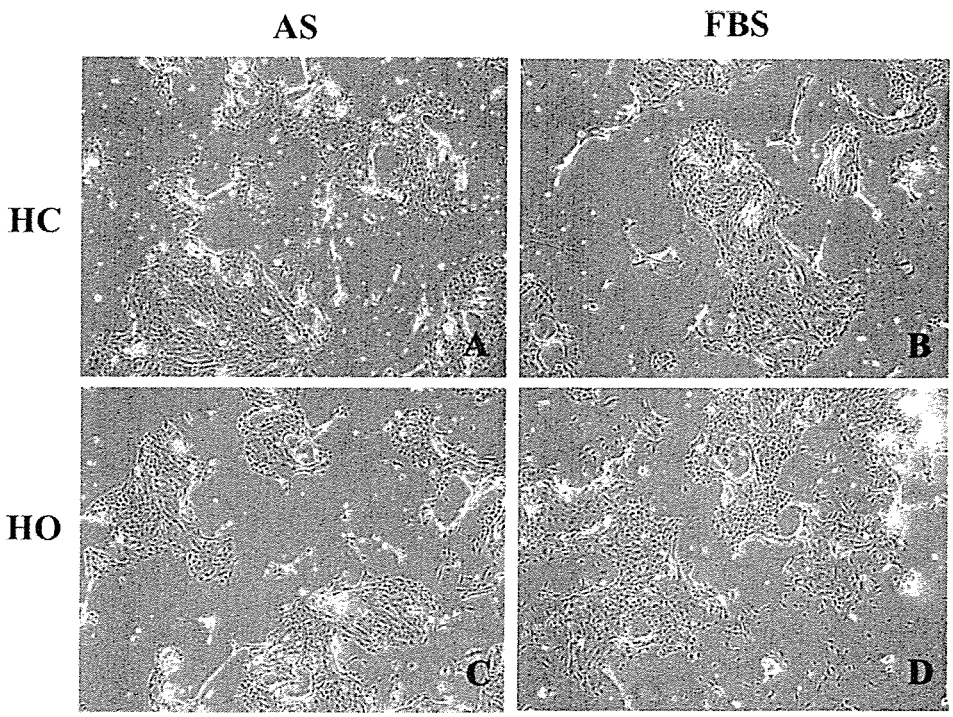

FIGURE 1. Representative phase contrast appearance of human corneal epithelial cells (A, B) and human oral epithelial cells (C, D) cultivated in AS- (A, C) and FBS-supplemented (B, D) media on day 6. In both AS- and FBS-supplemented media, human corneal and oral epithelial cells formed colonies consisting 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.

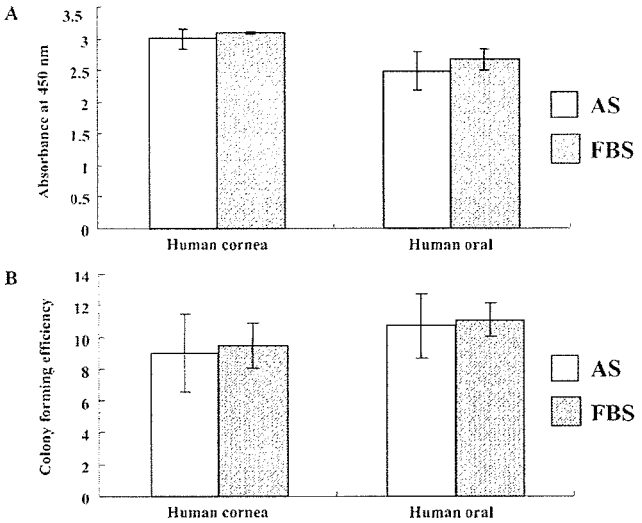

FIGURE 2. (A) BrdU ELISA cell proliferation assay of corneal and oral epithelial cells cultivated in AS- (n = 12) and FBS-supplemented (n = 12) media. The bars show the mean values of BrdU absorbance in each culture condition. Proliferation indices (PI) of human corneal epithelial cells were 3.00 ± 0.16 (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

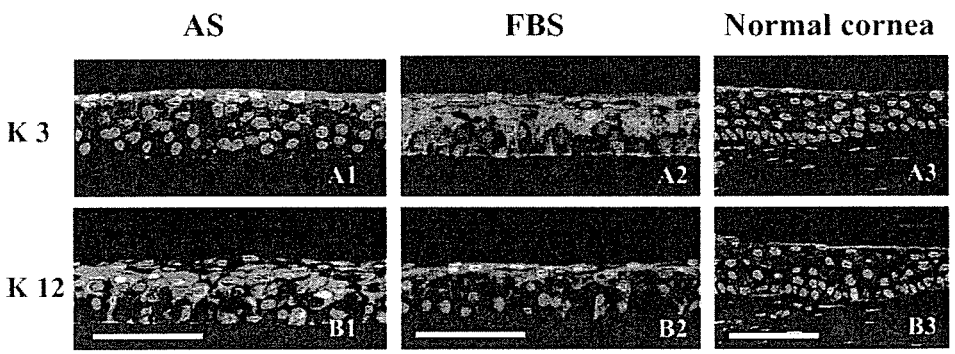

FIGURE 3. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (**A1**, **B1**) and FBS-supplemented (**A2**, **B2**) media, compared with normal in vivo cornea epithelium (**A3**, **B3**). In all 3 epithelia, cornea-specific keratins 3 (**A1-A3**) and 12 (**B1-B3**) 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.

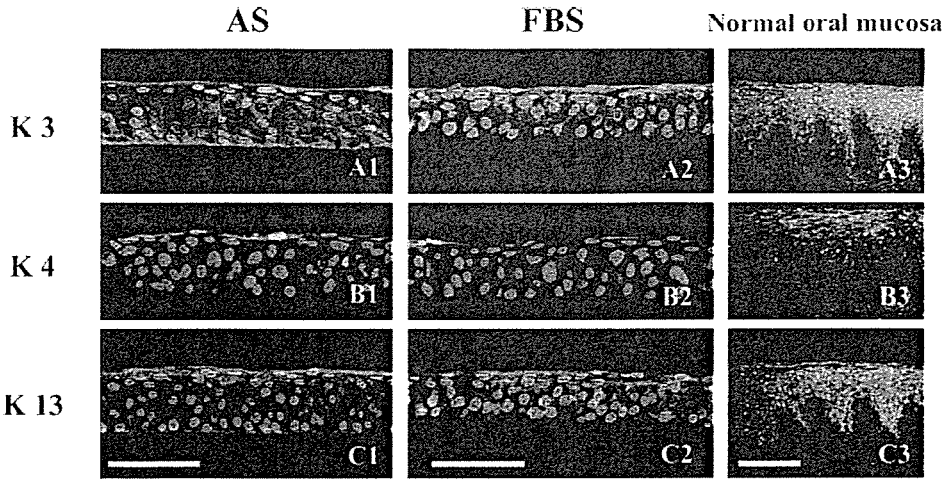

FIGURE 4. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (**A1–C1**) and FBS-supplemented (**A2–C2**) media, compared with normal in vivo oral epithelium (**A3–C3**). In normal oral epithelium, keratin 3 (**A3**) and keratin 13 (**C3**) were expressed in all epithelial 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.

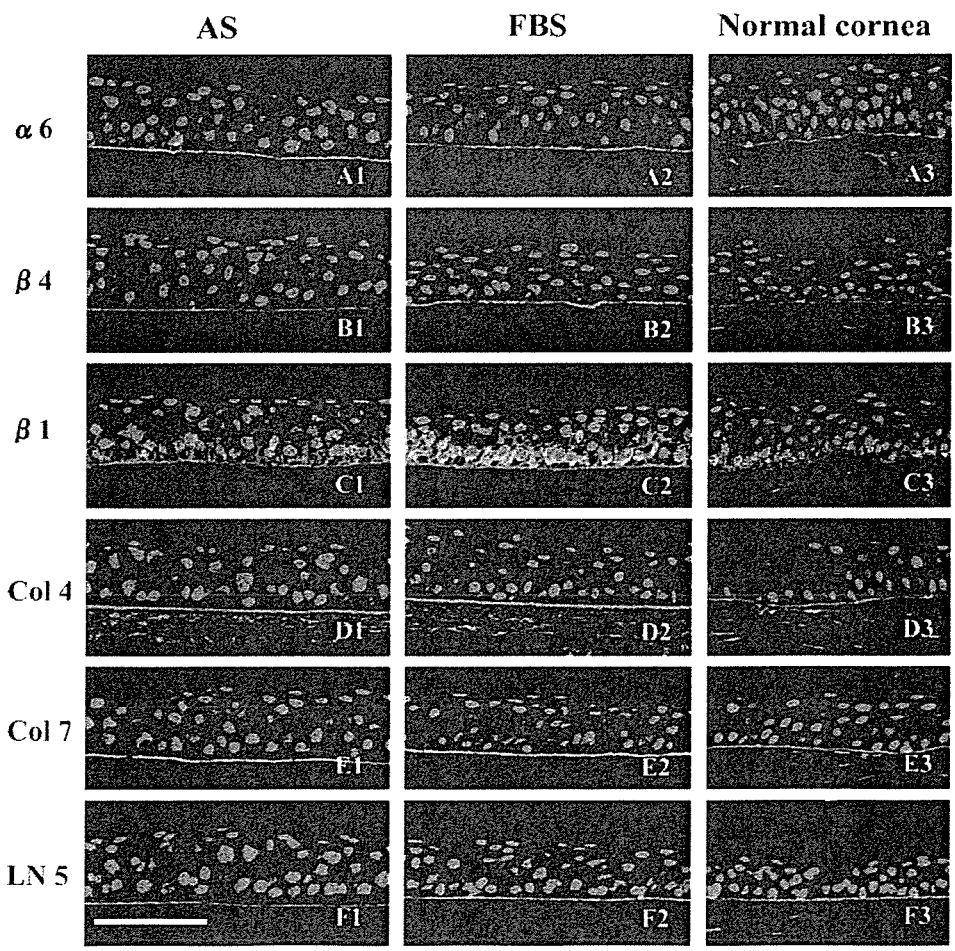

FIGURE 5. Representative immunohistochemical results of cultivated cornea epithelial sheets in AS- (**A1–F1**) and FBS-supplemented (**A2–F2**) media, compared with normal in vivo cornea epithelium (**A3–F3**). There was linear positive staining of integrin α6 (**A1–A3**), integrin β4 (**B1–B3**), collagen 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.

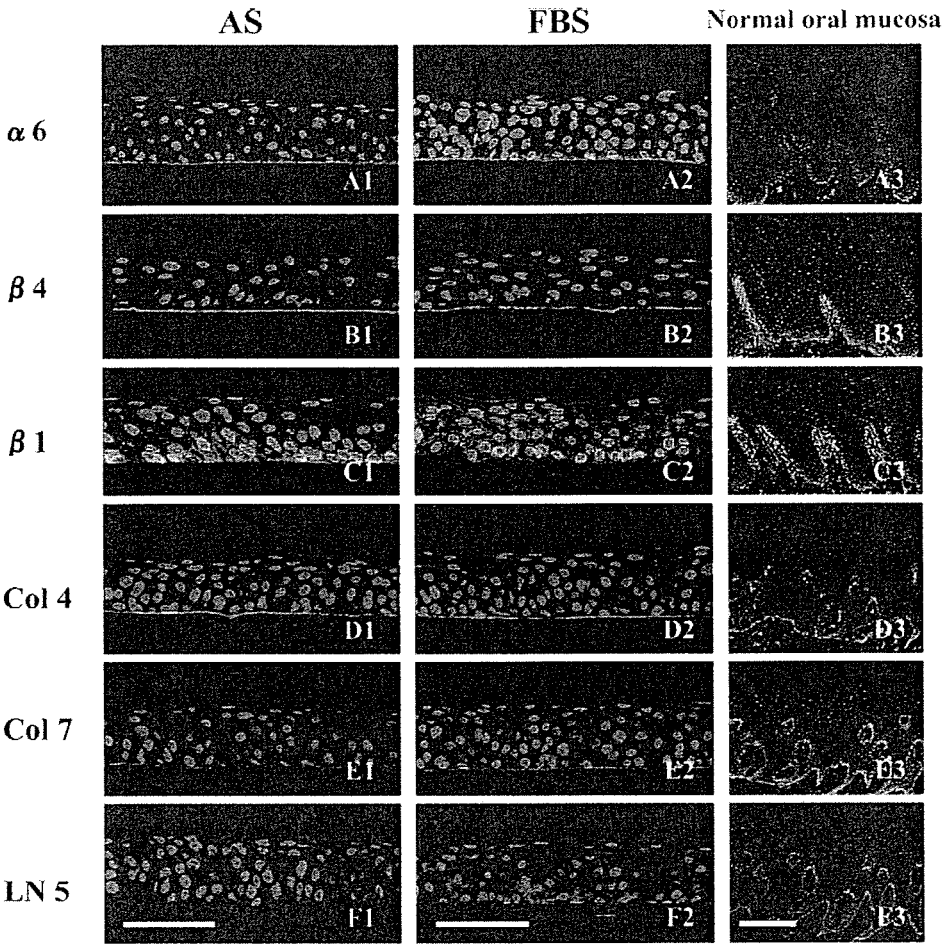

FIGURE 6. Representative immunohistochemical results of cultivated oral epithelial sheets in AS- (**A1–F1**) and FBS-supplemented (**A2–F2**) media, compared with normal in vivo oral epithelium (**A3–F3**). There was linear positive staining of integrin α6 (**A1–A3**), integrin β4 (**B1–B3**), collagen 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.

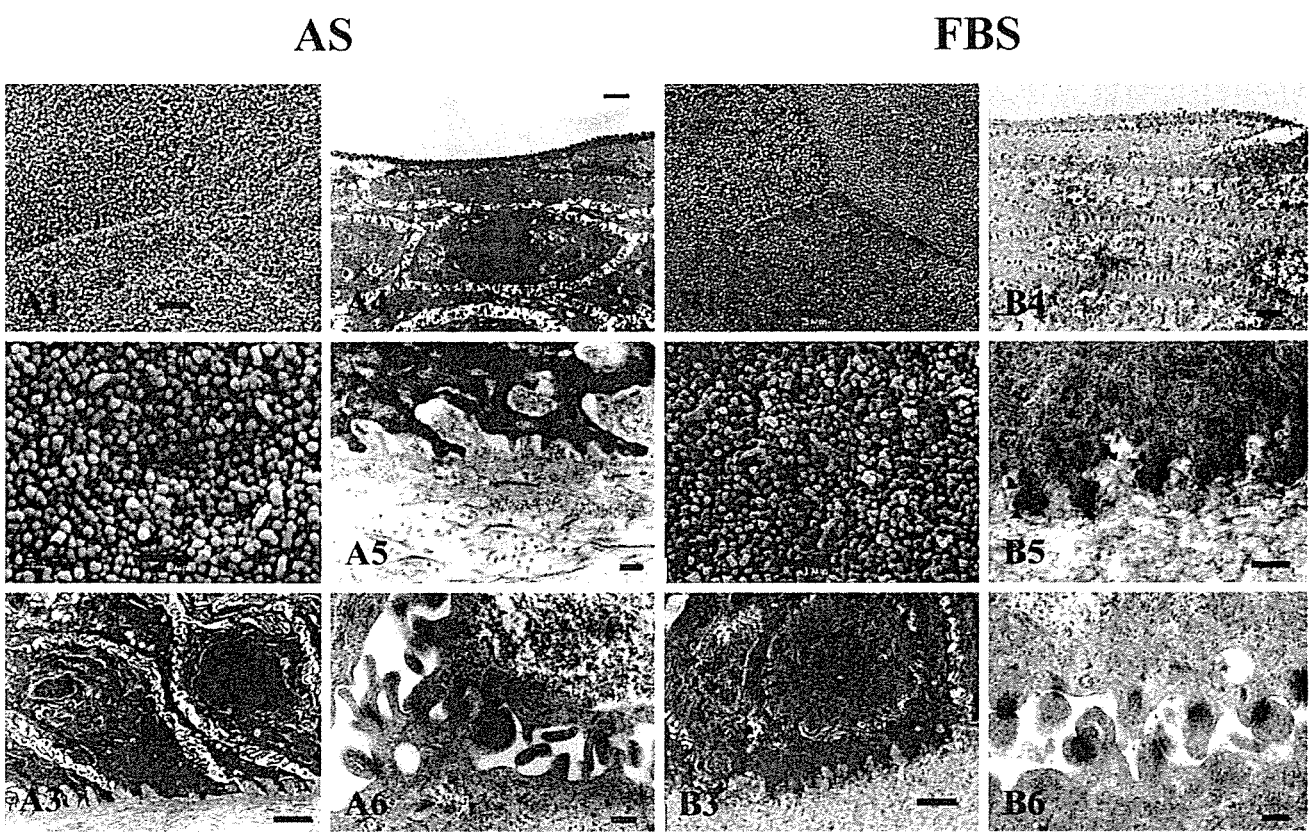

FIGURE 7. Scanning electron microscopic examination showed a continuous layer of flat squamous polygonal epithelial cells in the cultivated corneal epithelial cells when using AS (**A1**) and FBS (**B1**). These cells were closely attached to each other with tightly opposed cell junctions and distinct 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 $\alpha 6$ and $\beta 4$ 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.

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Clusters of Corneal Epithelial Cells Reside Ectopically in Human Conjunctival Epithelium

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Purpose. The ocular surface is covered by two biologically distinct epithelia: corneal and conjunctival. The expression of keratin12 (K12) is currently considered a hallmark of cornea-type differentiation. In the current study, the biological features of K12-positive cells in human bulbar conjunctival epithelium were examined.

Methods. Human conjunctival tissues were subjected to investigate the K12-positive cells in conjunctiva by immunostaining, in situ hybridization, Western blot analysis, reverse transcriptase-polymerase chain reaction (RT-PCR), and fluorescence-activated cell sorting (FACS). Gene expression profiling of these cells was performed with introduced amplified-fragment length polymorphism (iAFLP). To determine the presence of stem- or progenitor cells, immunostaining and colony-forming assays were performed.

Results. Western blot analysis, RT-PCR revealed that K12 was expressed in conjunctival epithelium. Immunostaining analysis showed that K12-positive cells reside mainly in clusters in conjunctival epithelium. FACS analysis showed that 0.2% to 1.7% of conjunctival epithelial cells collected from the inferior bulbar conjunctiva were K12 positive. iAFLP analysis revealed that the gene expression patterns of these cells were highly similar to that of corneal epithelial cells. p63 and ABCG2 were expressed beneath the K12-positive cells. Some colony-forming cells expressed K12.

Conclusions. The K12-positive cells appear to be ectopically residing, self-maintaining corneal epithelial cells in the conjunctival epithelium. (*Invest Ophthalmol Vis Sci.* 2006;47: 1359–1367) DOI:10.1167/iovs.05-1084

The ocular surface is covered by two different types of epithelia: the conjunctival and the corneal epithelium. Corneal epithelial cells are continuously supplied from the limbus where their stem cells reside.[1–5] Conjunctival epithelial stem cells were found primarily at the conjunctival fornix by colony-forming assay in humans[5] and by in vivo label-retaining experiments in rabbits[6] and mice.[7,8] More recently, time-lapse studies in green fluorescent protein (GFP) mice conjunctiva disclosed the uniform distribution of stem cells in the bulbar conjunctiva.[9] In addition, the mucocutaneous junction conjunctiva has been shown to contain stem cells that migrate toward the fornix.[10,11] Thus, the site where conjunctival epithelial stem cells reside remains controversial.

Besides their anatomic segregation, the two types of ocular epithelium possess unique tissue- and cytological properties. For example, conjunctival epithelium does, while corneal epithelium does not, contain mucin-secreting goblet cells.[12] Our previous gene expression analysis[13,14] disclosed that many genes are differentially expressed by these epithelia. Wei et al.[15] reported that rabbit conjunctival and corneal epithelial cells belong to two separate lineages. Based on these observations, corneal and conjunctival epithelial cells appear to be intrinsically different.

The current dogma is that the expression of K3/12 is thought to be a hallmark of epithelia with cornea-type differentiation[1,15–20] and to be indispensable for corneal epithelial homeostasis.[21] However, K3 is also expressed in other epithelia, including snout,[16] gingiva, and tongue[22] and palpebral conjunctiva.[6] Also, it has been reported that bovine bulbar conjunctival epithelial cells expressed trace amounts of K3 and were induced to express K3/12 by inoculation onto corneal basement membrane.[23] Similar findings were made on cultured rabbit conjunctival epithelial cells.[6] These reports strongly suggest that the actual expression patterns of K3/12 in ocular surface epithelium are not as clear cut as the current dogma.

We investigated expression of K12 in human conjunctival epithelium. We found that K12-positive cells are present in this tissue primarily as clusters and appear to possess cellular features highly similar to corneal epithelial cells. Furthermore, they seem to have their own stem- or progenitor cells. Based on our observations, we postulated that the K12-positive cells in conjunctival epithelium are ectopically residing corneal epithelial cells.

Methods

Human Samples

This study was approved by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine and was performed in accordance with the tenets of the Declaration of Helsinki.

Normal conjunctival tissues were obtained from otherwise healthy eyes at cataract or conjunctivochalasis surgery. The resected normal conjunctivae from the patients with cataract (n = 10; three men and seven women; mean age, 69.0 ± 13.1 years) were 3 × 3 mm^2 in size and located at inferior bulbar conjunctiva 5 mm distant from the surgical limbus. The resected conjunctivae from the patients with conjunctivochalasis (n = 10; three men and seven women; mean age, 71.6 ± 8.7 years) were 3 to 6 mm (vertical) × 15 mm (horizontal) and located at the inferior bulbar conjunctiva, at least 2 mm distant from the limbus. Prior informed consent was obtained from all subjects after a detailed explanation of the procedures. Cadaveric corneas were obtained from the Northwest Lions' EyeBank (Seattle, WA). Permission to use the donated corneas for research was obtained from all donor families.

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TABLE 1. List of Antibodies

Antibody (Clone Name)	Type of Antibody	Immunized Animal	Source	Dilution
CK3(AE5)	Mono	Mouse	PROGEN	×50
CK4(6B10)	Mono	Mouse	Novocastra	×200
CK12(sc-17098)	Poly	Goat	Santa Cruz	×100
CK12	Poly	Rabbit	NC	×100
CK13(KS-1A3)	Mono	Mouse	Novocastra	×200
MUC5AC(CLH2)	Mono	Mouse	Novocastra	×100
ABCG2(BXP-21)	Mono	Mouse	KAMIYA	×40
p63(4A4)	Mono	Mouse	Santa Cruz	×100

Mono: monoclonal antibody, Poly: polyclonal antibody; PROGEN, PROGEN Biotechnik GmbH, Heidelberg, Germany; Novocastra, Novocastra Laboratories Ltd, Newcastle, UK; Santa Cruz, Santa Cruz Biotechnology Inc., Santa Cruz, CA; NC, not commercially available; KAMIYA, Kamiya Biomedical Company, Seattle, WA.

Cryosectioning

Corneal and conjunctival tissues were embedded in OCT compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan) and snap frozen with liquid nitrogen. Sections were placed on glass slides for immunostaining and in situ hybridization or on slides (Penfoil; Leica Microsystems, Co., Ltd., Wetzlar, Germany) for laser microdissection.

Immunostaining

Sections or cells were dried and fixed at 4°C with Zamboni's fixative. Then they were incubated in blocking solution (1% BSA in 0.01 M PBS), incubated in the primary antibodies (Table 1), washed with 0.01 M PBS, incubated again with the corresponding fluorescence-labeled secondary antibody, immersed in mounting medium, and covered with coverslips.

Western Blot Analysis

Conjunctival tissue was stretched on a filter paper epithelial side up. With a spatula, the epithelium was mechanically scraped from the tissue with special care taken to avoid breaking the underlying stroma. The collected epithelium was lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.6 M KCl, 1% Triton X-100, and a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Penzberg, Germany). After centrifugation, pellets were solubilized in sample buffer containing 25 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.005% bromphenol blue (BPB). The samples were electrophoresed, transferred to a polyvinylidene difluoride (PVDF) membrane (HybondP; GE Healthcare, Piscataway, NJ) and immunostained with anti-K12 antibody (1:1000, sc-17098; Santa Cruz Biotechnology, Santa Cruz, CA).

Fluorescence-Activated Cell Sorting

The conjunctival epithelium was separated from underlying stroma with 1.2 U/mL dispase[24] and further disintegrated with 0.05% trypsin/EDTA. After fixation with 4% paraformaldehyde, the cells were incubated in a blocking buffer containing 0.1 M PBS and 0.5% BSA. After incubation in a permeabilization buffer (0.5% saponin in the blocking buffer), the cells were immunostained with anti-K12 antibody (1;100, sc-17098) or normal goat IgG. After washing in 0.1 M PBS, the cells were stained with Alexa488 anti-goat IgG (Invitrogen, Carlsbad, CA) and analyzed by fluorescence-activated cell sorting (FACS; FACSCaliber, BD Biosciences, San Jose, CA).

Fluorescence In Situ Hybridization

A specific region for K12 mRNA (nucleotide positions 1337-1792 in NM000223) was amplified and cloned into a T-overhang vector (pGEM-T Easy Vector; Promega, Madison, WI). After confirmation by sequencing, the plasmid was digested with restriction enzyme and used to prepare a sense or antisense digoxigenin (DIG)-labeled RNA probe.

Frozen conjunctival sections (10 μm) were fixed with 4% paraformaldehyde and incubated in a 0.2-μg/mL proteinase-K solution. Then, they were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (TEA; pH 8.0), washed with PBS, and dehydrated with a graded series of ethanol. After 30-minute air drying, they were incubated in hybridization buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10% dextran sulfate, 1× Denhardt solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 500 ng/mL salmon sperm DNA (Invitrogen), 0.5 mg/mL yeast tRNA (Roche Diagnostics), and 10 mM dithiothreitol [DTT] plus 10 ng/mL of the sense or antisense probe. After an 18-hour incubation at 60°C, the sections were washed twice at 52°C for 30 minutes in 0.5× SSC and 50% formamide and then washed twice for 15 minutes at room temperature in 0.2× SSC. After a 30-minute incubation in a blocking buffer, the sections were incubated in horseradish peroxidase (HRP)-labeled anti-DIG antibody (1:100; Roche Diagnostics) solution. After signal intensification by tyramide signal amplification (Biotin-TSA kit; Perkin-Elmer Life Sciences Inc., Boston, MA), the sections were incubated with Alexa488-labeled streptavidin (Invitrogen).

Reverse Transcriptase–Polymerase Chain Reaction and Real-Time PCR

RNAs were extracted from corneal or conjunctival epithelium, reverse-transcribed, amplified with primer pairs against the genes (Table 2), and electrophoresed in 2% agarose gels. Southern blot analysis was performed to validate the results.

Real-time PCR was performed to quantitate the relative gene expression of K12 and ribosomal RNA (for normalization) using a sequence-detection system (Prism 7000 Sequence Detection System; Applied Biosystems, Ltd. [ABI], Tokyo, Japan). (Sequences for these primers and internal probes were not disclosed.)

Laser Microdissection

For introduced amplified fragment length polymorphism (iAFLP) analysis, K12-positive and K12-negative cells were individually harvested from three individual conjunctivas (Fig. 1) by using a laser-microdissection device (AS LMD; Leica Microsystems).

Gene Expression Analysis by iAFLP

Corneal or conjunctival epithelial cells were mechanically peeled from five corneas or five conjunctivas. RNAs were extracted from these cells or from six microdissected samples (TRIzol reagent; Invitrogen).

Comprehensive gene expression profiles were examined with the iAFLP method of Kawamoto et al.[25] slightly modified. Briefly, double-stranded cDNA was synthesized with a pUC119-based vector primer, as described previously,[26] and digested with *Mbo*I for subsequent adaptor ligation. Small aliquots (approximately one-sixth) of all digested cDNAs were pooled to obtain a reference sample to connect the data among the different sample sets. Each of the cDNA samples, including the reference sample, was ligated with an individual length polymorphic adaptor (TTnew33-TTnew45 adaptors for individual samples and TTnew48 adaptor for the reference sample). Five different cDNA samples and the reference sample were pooled to make four sample sets in total. After PCR amplification with AntVpPst and T7revBam primers, the four sample sets were digested with *Bam*HI, ligated with the T7-3000 adaptor, and amplified by PCR with a fluorescent-labeled MA20 primer and a gene-specific primer. Gene-specific primers (288 genes) were designed to analyze genes that were dominantly and/or specifically expressed in corneal epithelial cells (for gene selection, we referred to the Bodymap database; http://bodymap.ims.u-tokyo.ac.jp/). Each amplified product was electrophoresed on a fluorescence autosequencer (ABI3100 DNA analyzer; ABI) and the results were analyzed on computer (Genescan and Genotyper software; ABI). The resultant gene expression data were further analyzed with Cluster and Treeview software.[27] All oligomers and adaptors except for gene-specific primers used in iAFLP analysis are listed in Table 2.

TABLE 2. Oligomers

Category	Oligomer	Sequence
RT-PCR	K3_forward	CTGTCAGCATCTCCGTGGT
	K3_reverse	GCACTGAAGCCACCTCCTAA
	K4_forward	AATGTCTGGAGAATGCCAGAG
	K4_reverse	CGTCTCTTGTTCAGGGTGGT
	K12_forward	AAGGTGATGGTTTGGAGGAA
	K12_reverse	AATCATGGGGCAGATCTTGT
	K13_forward	GATCCAGGGACTCATCAGCA
	K13_reverse	AAGGCCTACGGACATCAGAA
	TKT_forward	CTGCTTCATCCGGACCAG
	TKT_reverse	CACACTTCATACCCGCCCTA
	TGFBI_forward	ACCTCAGGAAAGAGGGGATG
	TGFBI_reverse	GGCTGGATTGCTTGATTCAT
	ALDH3_forward	TTGCAGAGACATCCAGTGGT
	ALDH4_reverse	TTGGTCTAGAAAGGGGTGGA
	CTSL2_forward	TTGCTAATGACACTGGCTTCA
	CTSL3_reverse	TGGATCCTCAATGATTCAACTG
	GJA1_forward	GTACCAAACAGCAGCGGAGT
	GJA2_reverse	CAGTTTGGGCAACCTTGAGT
SB	K3_probe	B-AGGTGGCTATGGAGGAGGTT
	K4_probe	B-CAGTGTCTCTGGCAGTTCCA
	K12_probe	B-TGAATGGTGAGGTGGTCTCA
	K13_probe	B-CAGTGAGATGGAGTGCCAGA
	Biotin N20	B-NNNNNNNNNNNNNNNNNNNN
ISH	K12_ISH_forward	GAAGGTGATGGTTTGGAGGAA
	K12_ISH_reverse	TTCCGGGTTACCAGAAGAAA
iAFLP	T7revBam	AGAGGGATATCACTCGGATCCAT
	AntVpPst	GCCAAGCTTGCATGCCTGCATTTTTTT
	TTnew33	AGAGGGATATCACTCGGATCCATCAGTCAGGAT
	TTnew36	AGAGGGATATCACTCGGATCCATATCCAGTCAGGAT
	TTnew39	AGAGGGATATCACTCGGATCCATACTATCCAGTCAGGAT
	TTnew42	AGAGGGATATCACTCGGATCCATTCTACTATCCAGTCAGGAT
	TTnew45	AGAGGGATATCACTCGGATCCATCAATCTACTATCCAGTCAGGAT
	TTnew48	AGAGGGATATCACTCGGATCCATACTCAATCTACTATCCAGTCAGGAT
	NH1400P	P-GATCATCCTGACTG-NH2
	T7_3000	GCACTATAGGGAGATTACTTTAGGACTGAC
	NH14_rev	P-GATCGTCAGTCCTA
	MA20	F-GCACTATAGGGAGATTACTT

For the 3′ or 5′ modification, B means biotinylation, P means phosphorylation, NH2 means amino-linker, and F means fluorescent dye (6-Fam). Length polymorphic adaptors were made by pooling an equimolar amount of NH1400 oligomer and one of TTnew (TTnew33-TTnew48) oligomers. The T7_3000 adaptor was made by pooling an equimolar amount of T7_3000 and NH14_rev oligomers. All oligonucleotides were synthesized by Promega.

Virtual Northern Blot

Full-length cDNAs were amplified by a cDNA synthesis kit (Super Smart PCR; BD-Clontech, Mountain View, CA). The cDNAs were electrophoresed, transferred to a nylon membrane, and hybridized with biotin-labeled probes.

Colony-Forming Assay

A colony-forming assay was performed as described previously.[5] Briefly, conjunctival epithelial cells were enzymatically dissociated and seeded on a feeder layer of MMC-treated 3T3 cells. After 4 to 5 days, the cells were fixed with Zamboni's fixative and subjected to immunostaining.

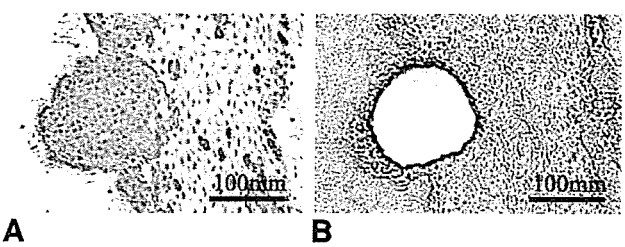


FIGURE 1. Microdissection of K12-positive and K12-negative cells residing in conjunctival epithelium. Conjunctival K12-positive or -negative cells were selectively collected by laser microdissection (**B**) by inspecting contiguous K12-immunostained sections (**A**).

Cell Culture

Conjunctival epithelial cells were organotypically cultured on an human amniotic membrane, according to a previously described method.[28,29] After 7 days of culture at the air–liquid interface, the cultured conjunctival epithelial sheet was embedded in OCT compound, cryosectioned, and subjected to immunostaining.

Image Acquisition

All fluorescent images were acquired with a confocal laser (TCS SP2 AOBS; Leica Microsystems) or a fluorescent microscope (Olympus Corp., Tokyo, Japan). All chemiluminescent images were acquired in an intelligent dark box (VersaDoc 5000; Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS

K12-Positive Cells in Conjunctival Epithelium

Immunostaining analysis using five donor tissues extending from the peripheral cornea to the bulbar conjunctiva revealed

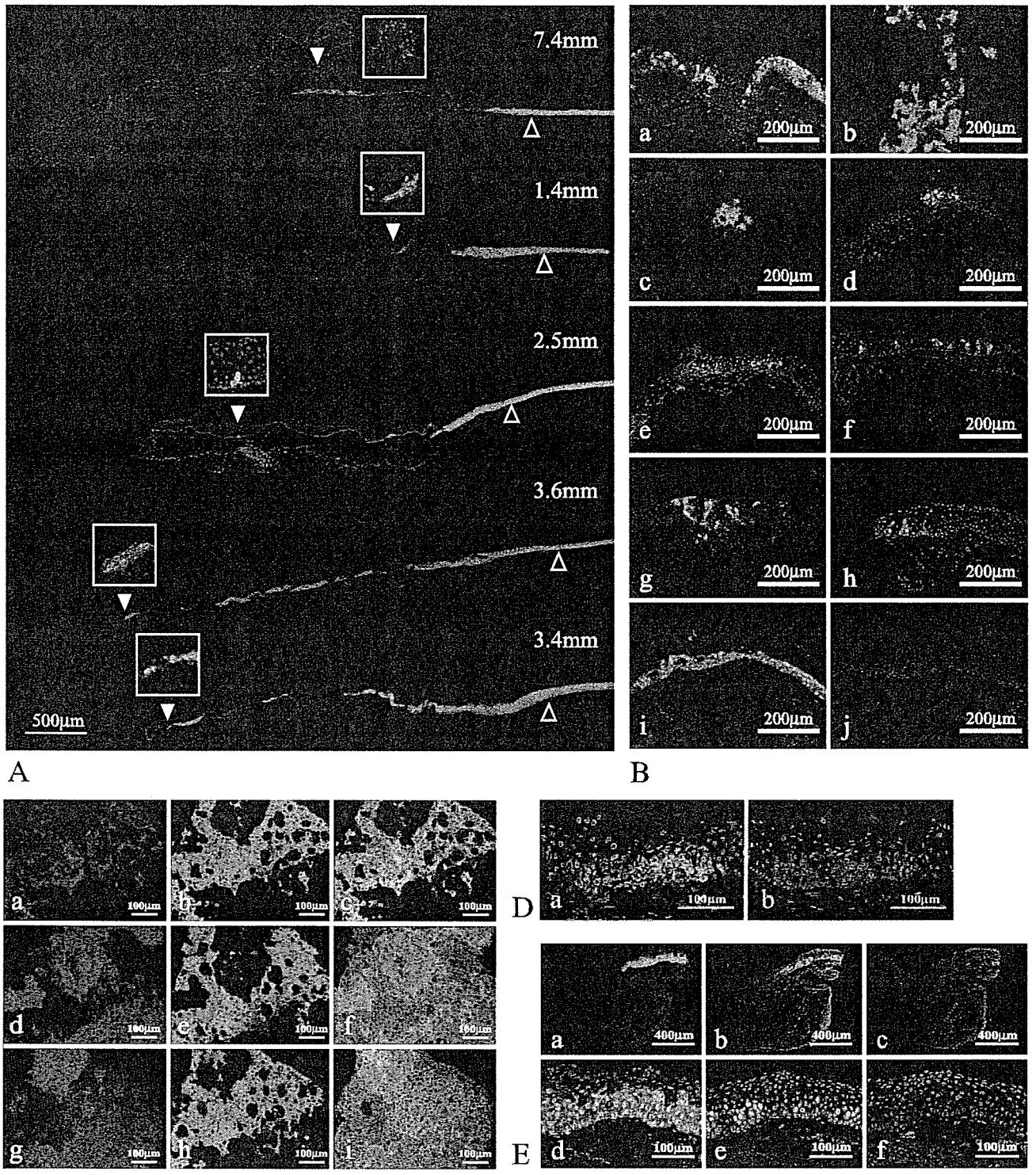


FIGURE 2. Tissue localization of K12-positive cells in conjunctival epithelium. (**A**) Expression of K12 in corneoscleral tissues. Corneoscleral tissues were immunostained with anti-K12 antibody (*green*) and counterstained with propidium iodide (*red*). Distance from the end of the Bowman's membrane (*open arrowhead*) to the most distal K12-positive cell cluster (*filled arrowhead*) was shown at the *right* of each sample. (**B**) Expression of K12 in conjunctival epithelium. Conjunctival tissues were immunostained with anti-K12 antibody (*green*) and counterstained with propidium iodide (*red*). Nine samples (**Ba–i**) exhibit K12-positive cell cluster(s) while 1 sample (**Bj**) does not. (**C**) Expression of the four abundant keratins in conjunctival epithelium. Conjunctival tissues were double-immunostained against K3 (**Ca**) and K12 (**Cb**), K4 (**Cd**), and K12 (**Ce**), or K13 (**Cg**) and K12 (**Ch**). Note that K3 and K12 colocalize (**Cc**), whereas expression of K4 and K12 (**Cf**) and that of K13 and K12 (**Ci**) are almost mutually exclusive. (**D**) K12 expression using two different antibodies. Conjunctival tissue was immunostained against K12 (*green*) using either goat polyclonal antibody (**Da**) or rabbit polyclonal antibody (**Db**). Note that these two different K12 antibodies produced consistent immunostaining results. (**E**) Expression of K12 mRNA in conjunctival epithelium. The photographs were taken at low (**Ea–c**) and high (**Ed–f**) magnification. Images of conjunctival tissues processed by immunostaining (**Ea**, **Ed**) or fluorescent in situ hybridization (**Eb**, **Ee**; antisense probe, **Ec**, **Ef**; sense probe) demonstrate the consistent expression pattern between K12 protein (*green*) and its mRNA (*green*).

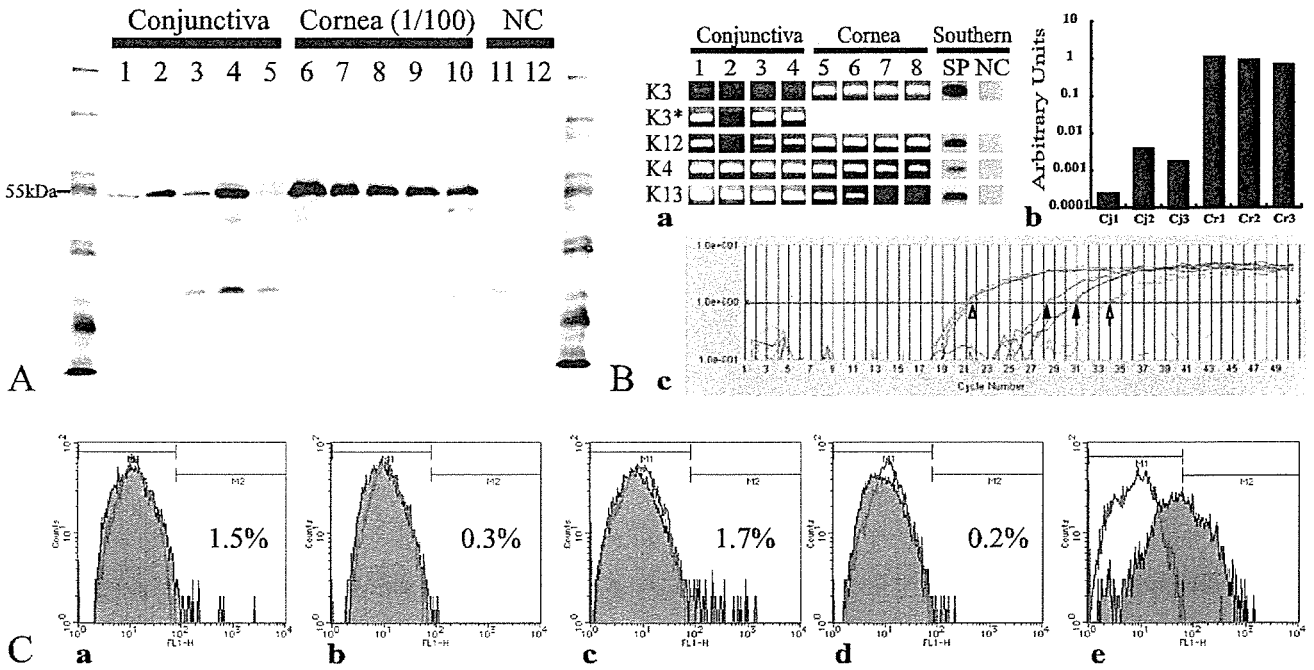


FIGURE 3. Expression of K12 in conjunctival epithelium. (**A**) Expression of K12 protein in conjunctival epithelium (Western blot analysis). NC denotes negative control. Protein samples prepared from the insoluble fraction of conjunctival (*lanes 1* to *5*, *11*) or corneal (*lane 6* to *10*, *12*) epithelial lysates were electrophoresed, transferred, and immunostained against K12 (*lanes 1* to *10*) or normal goat IgG (*lanes 11*, *12*). Note that the loaded protein amount of corneal samples was reduced to 1:100 of that of conjunctiva to avoid signal quenching. (**B**) Expression of the keratin genes detected by RT-PCR and real-time PCR. (**Ba**) The expression of K3, -4, -12, and -13 was analyzed by RT-PCR and validated by Southern blot analysis. Expression of K3 was not detected in conjunctival epithelium with a normal three-temperature thermal setting but was detected under a touchdown thermal condition (*). (**Bb**) Quantitative expression of K12 in corneal (Cr1, Cr2, Cr3) and conjunctival (Cj1, Cj2, Cj3) epithelia by real-time PCR analysis. The expression level of K12 in conjunctival epithelium was no more than 1% of that in corneal epithelium. (**Bc**) The kinetics of K12 gene amplification were monitored by real-time PCR. (*Open arrowhead*) Three corneal samples; (*filled arrowhead*) Cj2; (*filled arrow*) Cj3; (*open arrow*) Cj1. *Red horizontal line*: the threshold line. (**C**) FACS analysis of K12-positive cells in conjunctival epithelium. Conjunctival (**Ca–d**) and corneal (**Ce**) epithelial cells were dispersed by enzymatic dissociation, fixed, and immunostained against K12. In the conjunctiva, K12-positive cells (*purple*, M2 region) comprised approximately 0.2% to 1.7% of total analyzed cells. The positive–negative cutoff line was defined according to the signal distribution of the isotype-negative control (*red line*).

the presence of K12-positive cell clusters in conjunctiva at a site far from the limbus (Fig. 2A). On average, the farthest K12-positive cell cluster in each sample was located at a distance of approximately 3.7 ± 2.3 mm from the end of Bowman's membrane. Among these, the farthest was 7.4 mm from the end of Bowman's membrane, a region that can be considered to be the bulbar conjunctival or the conjunctival fornix. In the limbal area, the expression pattern of K12 was almost the same as that in cornea, except that intermediate to superficial layers tended to retard K12 expression. We examined the existence of such cell clusters in conjunctival epithelia of 10 different subjects. All conjunctivae, except that of one subject (Fig. 2Bj), exhibited K12-positive cells and clusters in the conjunctival epithelium (Fig. 2B). Double-immunostaining analysis against K4 and K12 revealed that these keratins were expressed in a mutually exclusive manner (Figs. 2Cd–f), suggesting that these differentially stained cells have properties different from each other. We further examined the expression of K3 and K13, known to form a heterodimer with K12 and K4, respectively. As expected, the expression patterns for K12 and K3 were very similar (Figs. 2Ca–c). In contrast, K12 and K13 (Figs. 2Cg–i) presented an image that was similar to the images produced by double-immunostaining against K4 and K12.

The antibody we used (sc-17098) was raised against the N-terminal partial peptide sequence. To examine the possibility that this antibody reacted with other molecules, immunostaining was again performed with a different K12 antibody.[17] The hypothetical cross-reactivity was almost completely abolished, as this antibody yielded an expression pattern very similar to that obtained with the other antibody (Fig. 2D).

Tissue localization of K12 mRNA, analyzed by in situ hybridization against sections contiguous with immunostained sections, demonstrated a tissue distribution pattern that was highly consistent with that of K12 protein (Fig. 2E). This finding strongly supported our immunostaining results. Western blot analysis demonstrated that conjunctival epithelial extracts produced a faint but specific band for K12 (Fig. 3A). RT-PCR analysis revealed that K12 mRNA was expressed in both corneal and conjunctival epithelium (Fig. 3B). However, real-time PCR analysis disclosed that the expression level of K12 mRNA in conjunctival epithelium was no more than 1% of that in corneal epithelium (Fig. 3B). FACS analysis also revealed that 0.2% to 1.7% of the conjunctival epithelial cells collected from the inferior bulbar conjunctiva was K12 positive (Fig. 3C). Taken together, despite individual variations, our findings suggest that as many as 1% of conjunctival epithelial cells seem to be K12 positive.

As some K12-positive cell clusters were located at quite a distance from the limbus, we hypothesized that these clusters are physiologically independent and spatially segregated from this region. To rule out the possibility that they are simply part of an extended limbal epithelium, we first looked for goblet cells, thought to be present only in conjunctival epithelium, in the vicinity of the K12-positive cell clusters. Double-immunostaining analysis against K12 and Mus5AC clearly demonstrated some MUC5AC-positive goblet cells very close to the K12-positive cell clusters (Fig. 4A). Next, we carefully inspected the tissue localization of the K12-positive cells in a series of contiguous sections. We found that some K12-positive clusters existed as solitary islands in conjunctival epithelium (Fig. 4B).

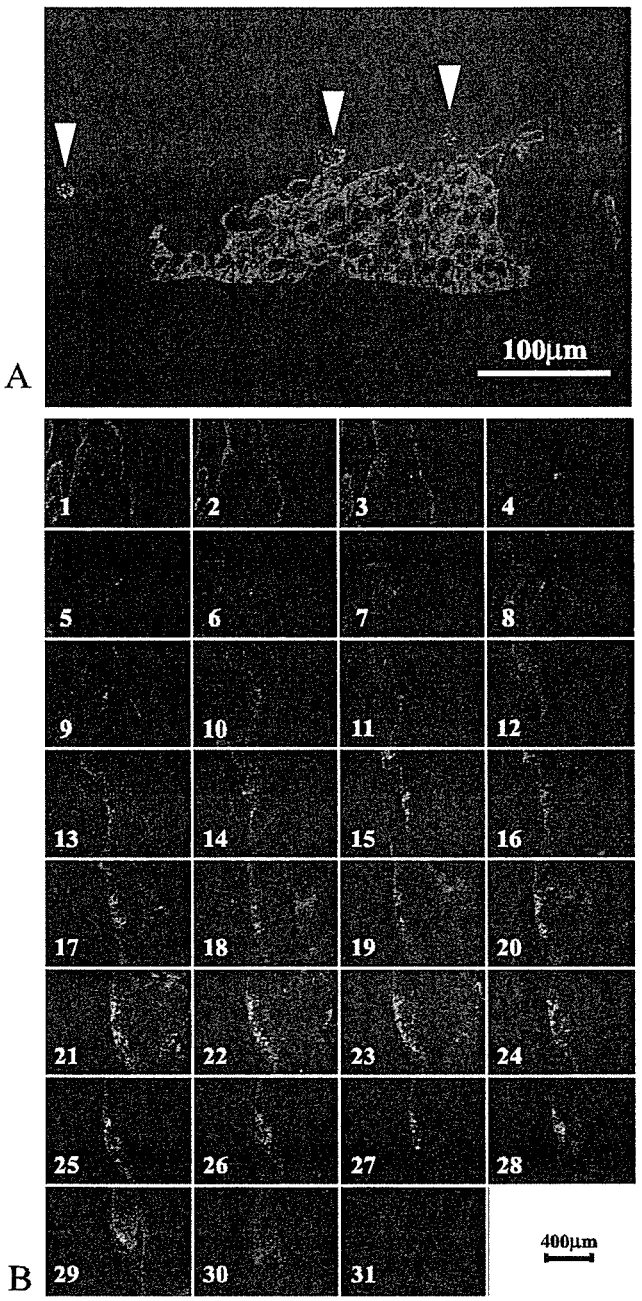


FIGURE 4. Segregation of conjunctival K12-positive cell clusters from limbal epithelium. (**A**) Expression of K12 and Muc5AC in conjunctival epithelium. Conjunctival tissue was immunostained against K12 (*green*) and Muc5AC (*red, arrowhead*) to demonstrate the spatial proximity of the conjunctival K12-positive cell clusters and goblet cells. (**B**) Isolated K12-positive cell cluster in conjunctival epithelium. The photographs show a series of contiguous sections to demonstrate that a conjunctival K12-positive cell cluster exists as a solitary island. Note that photographs 2 and 29 represent both edges of the K12-positive cluster.

Similarity of K12-Positive Cells in Conjunctival Epithelium to Corneal Epithelial Cells

As gene expression patterns vary significantly with tissue or cell type, we performed gene expression analysis using iAFLP to assign the K12-positive cells in the conjunctiva to the proper type of ocular surface epithelium. Of the 288 genes we examined, 185 could be analyzed; others could not, possibly due to improper primer sequences. Cluster analysis of the iAFLP data clearly demonstrated that the gene expression profiles of the K12-positive cells in the conjunctiva were similar to those of corneal epithelial cells (Figs. 5A, 5B). Among the genes examined here, 25 genes exhibited apparently different expression patterns (Fig. 5B) between conjunctival and corneal epithelium. Especially, TKT[30] and ALDH3[31] are known as dominant proteins in the cornea. Of note, *TGFBI* (keratoepithelin), a gene involved in hereditary corneal dystrophies,[32] was highly expressed both in corneal epithelial cells and conjunctival K12-positive cells. These data were further validated by RT-PCR (Fig. 5C) and virtual Northern blot analysis (Fig. 5D). The results strongly suggest that the K12-positive cells in the conjunctiva possess properties identical or very similar to those of corneal epithelial cells.

Stem Cells Associated with the K12-Positive Cells in the Conjunctiva

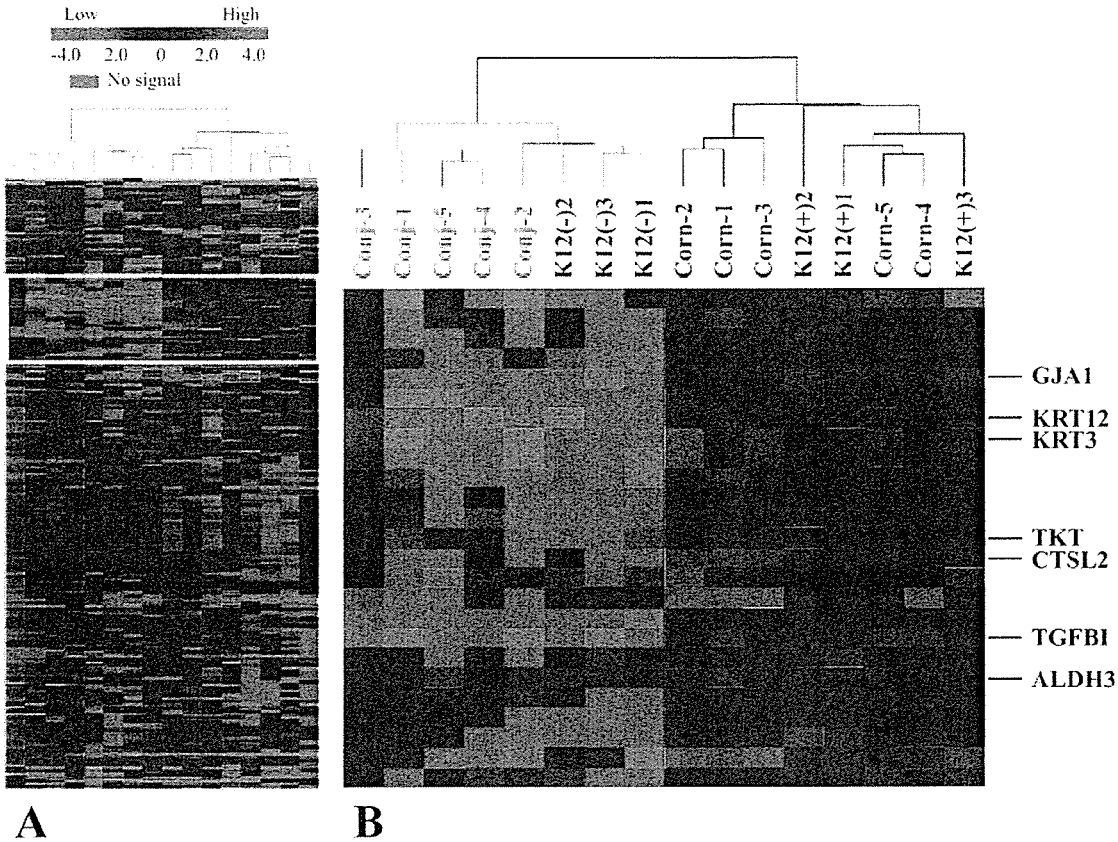
If the K12-positive cells in the conjunctiva are not derived from the limbus, where do these cells come from? We hypothesized that their stem cells reside just beneath them. Therefore, we looked for the expression of stem/progenitor cell markers around K12-positive cell clusters in the conjunctival epithelium. Among several putative markers for limbal basal stem cells,[33] we examined K12,[34] p63,[35,36] and ABCG2.[37] Some basal cells under the K12-positive cell clusters did not express K12 (Fig. 6A), implying that they were the stem cells of the overlying K12-positive cell cluster. Also, some basal-to-suprabasal cells beneath the K12-positive cell cluster expressed p63 (Fig. 6Aa) and ABCG2 (Fig. 6Ab), implying that these cells are stem/progenitor cells of the overlying K12-positive cell cluster. To test this hypothesis further, we investigated the colony-forming activity of these cells. As a result, some of the colonies expressed K12 (Fig. 6B), indicating that such K12 positive colonies are stem/progenitor cells of the K12-positive cells residing in conjunctiva. Then, we tested whether the K12-positive cells in the conjunctiva can be maintained after organotypic culture. We identified K12-positive cells in cultivated conjunctival epithelial sheets (Fig. 7).

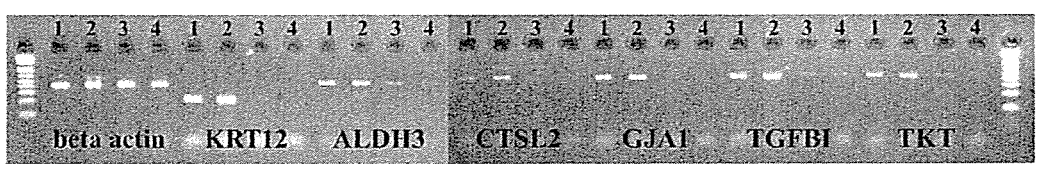
DISCUSSION

Immunostaining analysis clearly demonstrated the existence of K12-positive cells in human conjunctival epithelium. The results of Western blot analysis, in situ hybridization, FACS, and RT-PCR analyses further supported this observation. Moreover, gene expression analysis by iAFLP strongly suggests that the K12-positive cells in the conjunctiva possess properties highly similar to those of corneal epithelial cells. In addition, the K12-positive cells in the conjunctiva appeared to be maintained by their own stem or progenitor cells. Based on these results, we postulate that these cells are ectopically residing corneal epithelial cells self-maintained in the conjunctiva.

The most important issue in this study appears to be whether the K12-positive cells in conjunctiva are linked to corneal epithelial stem cells residing in the limbal basal layer. Our data strongly suggest that these cells are self-maintained in conjunctiva and are independent of the limbal basal stem cells. However, the possibility that limbal epithelium extends to such a distant region cannot be completely ruled out. Some radially sectioned corneoscleral tissues (Fig. 2A) demonstrate contiguous K12-positive cells from the limbus, implying this possibility. Investigation of whole ocular surface epithelium from cadaveric donors would shed light on this question.

Data derived from animal experiments led to the classic concept of conjunctival epithelial transdifferentiation—that is, conjunctival epithelial cells can become corneal epithelial cells under certain conditions,[38–41] thereby making it possible for eyes with total limbal failure to recover completely and exhibit





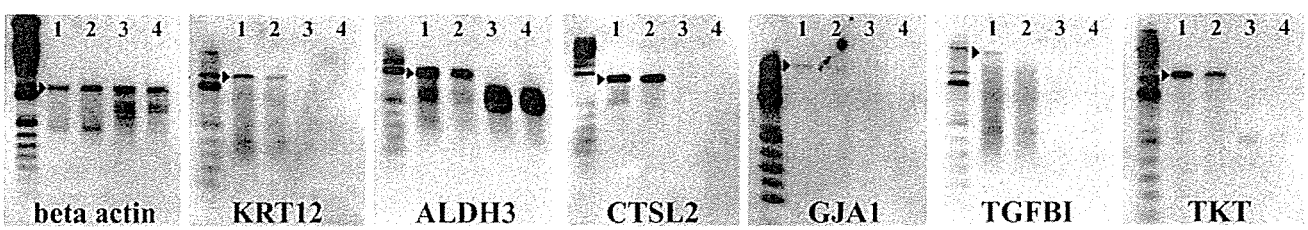


FIGURE 5. Gene expression profiling of the K12-positive cells in conjunctiva. Gene expression data on 185 genes from 16 samples were analyzed by hierarchical clustering. The 16 samples comprised corneal epithelial cells from five subjects (Corn-1–Corn-5), conjunctival epithelial cells from five different subjects (Conj-1–Conj-5), and laser microdissected K12-positive (K12(+)1–K12(+)3) and K12-negative (K12(−)1–K12(−)3) cells from three different conjunctivae. Each row represents an individual gene and each column an individual sample. The data were log-transformed (base 2) and centered in row-direction by subtracting the median observed value (log space). The data are depicted according to the color scale (log space) shown at *top left*. *Gray* data indicate that the electrophoresis data for the row were under the cutoff. (**A**) Whole image of two-dimensional hierarchical clustering of 185 genes across 16 samples. The horizontal hierarchical trees show the degree of similarity in the gene expression pattern among the 16 samples. Note that the 16 samples are clearly divided into two groups (*red* and *blue* trees). The area demarcated in *yellow* includes genes with expression that was significantly different in these two groups. (**B**) Differentially expressed genes between corneal and conjunctival epithelium . The color-coded matrix is a zoomed image of the area demarcated in *yellow* in (**A**). At the right, some well-known genes are represented by their symbols: *GJA1*, gap junction protein; α1 (connexin43); *KRT3*, keratin3; *KRT12*, keratin12; *TKT*, transketolase; *CTSL2*, cathepsinL2; *TGFBI*, beta IgH3; *ALDH3*, aldehyde dehydrogenase 3. Note that microdissected K12-positive samples manifest gene expression patterns highly similar to those of corneal epithelial cells. (**C**) Validation of the iAFLP results by RT-PCR. *Lanes 1, 2*: corneal epithelium, *lane 3, 4*: conjunctival epithelium. All genes, except for the β-actin gene, demonstrate dominant expression in corneal epithelium. All amplicons were confirmed by sequencing analysis. (**D**) Validation of the iAFLP results by virtual Northern blot. *Lane 1, 2*: corneal epithelium, *lane 3, 4*: conjunctival epithelium. Equal amount of amplified cDNAs were electrophoresed and hybridized. Each *arrowhead* indicates a band of authentic full-length cDNA of each gene.

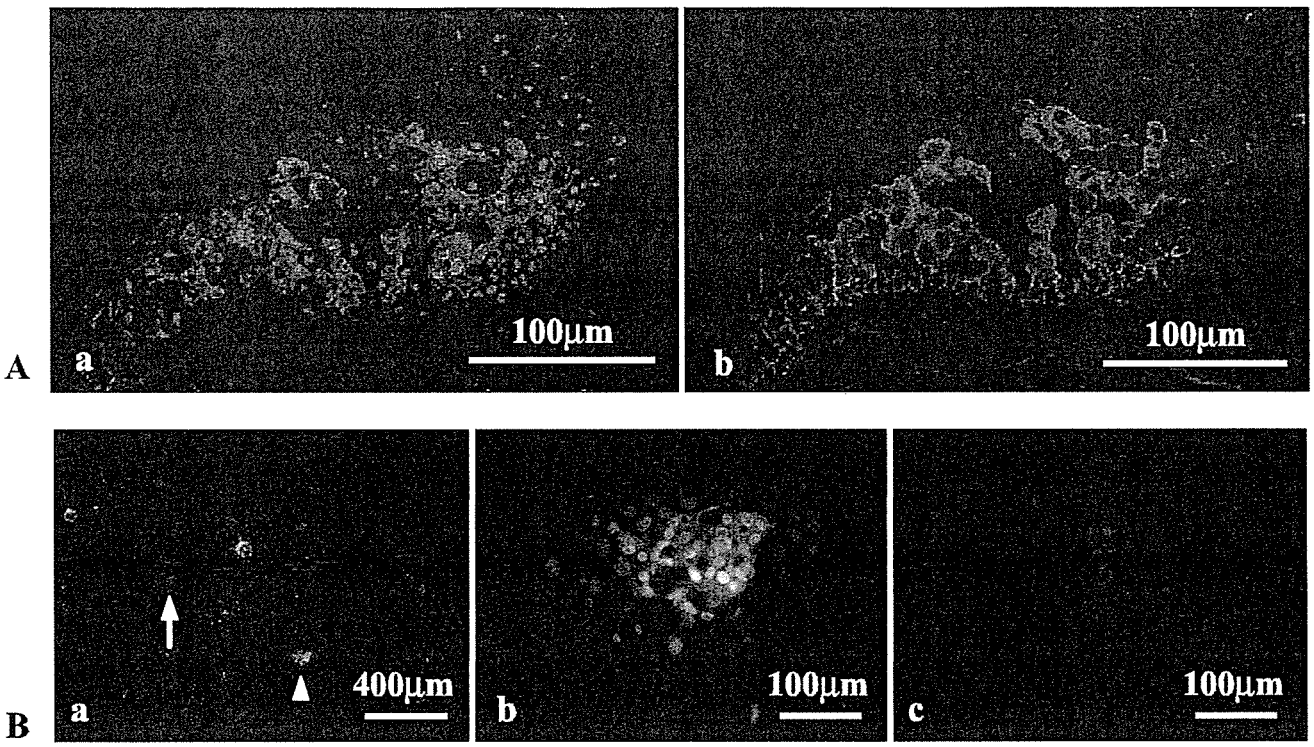


FIGURE 6. Presence of stem cells for conjunctival K12-positive cells. (**A**) Expression of stem cell markers in conjunctival K12-positive cell clusters. Conjunctival tissue was double-immunostained against K12 (*red*) and p63 (**Aa**, *green*) or K12 (*red*) and ABCG2 (**Ab**, *green*) and then counterstained with 4′,6′-diamino-2-phenylindole (DAPI; *blue*). Note that basal cells beneath the conjunctival K12-positive cell cluster are devoid of K12. (**B**) Expression of K12 by colony-forming cells of conjunctival epithelium. Conjunctival epithelial cells were dispersed by enzymatic digestion and seeded on MMC-treated 3T3 cells. After colonies became obvious, the cells were immunostained against K12 (*green*). (**Ba**) Some colonies expressed K12 (*arrow*), whereas others did not (*arrowhead*). (**Bb**) Zoomed image of the K12-positive colony identified by the *arrowhead* in (**Ba**). (**Bc**) The K12-negative colony identified by the *arrow* in (**Ba**).

a transparent cornea. Although clinical studies provided evidence in support of the transdifferentiation hypothesis[12] in humans, other animal experiments and data based on biochemical studies appeared to render this concept invalid,[13–17] because neither regenerating conjunctival epithelium covering the cornea nor organotypically cultured conjunctival epithelium exhibited cornea-specific phenotypes; rather, the phenotype was that of conjunctival epithelium. Based on our results, we postulate that during the epithelial regenerating process in patients with compromised limbal stem cells, ectopically residing corneal epithelial cells in the conjunctival epithelium migrate, cover the denuded cornea, and exhibit bona fide corneal epithelial properties. In this sense, the transdifferentiation concept would be incorrect from a cytological but correct from a clinical perspective.

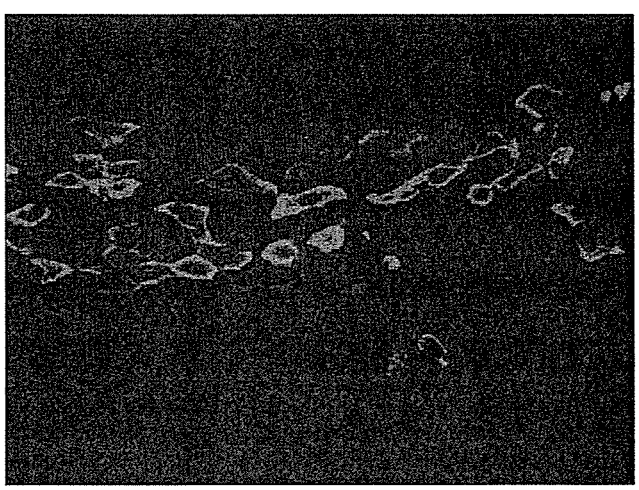


FIGURE 7. Expression of K12 in organotypically cultured conjunctival epithelium. Organotypically cultured conjunctival epithelium was immunostained against K12 (*green*) and then counterstained with 4′,6′-diamino-2-phenylindole (DAPI; *blue*).

In summary, ours is the first study to demonstrate clearly the existence of K12-positive cells in in vivo human conjunctival epithelium. We identified their cellular features by comprehensive gene-expression analysis and found them to be similar to the features of corneal epithelial cells. Moreover, K12-positive cells appear to have their own stem or progenitor cells. We submit the hypothesis that these cells are ectopically residing corneal epithelial cells and that they are self-maintained, even in conjunctival epithelium. Our preliminary findings that K12-positive cells exist in organotypically cultured conjunctival epithelium suggest that these cells can be maintained during the culture process. Sorting of these cells by FACS may allow us to generate cultured corneal epithelial sheets from conjunctiva. Studies are under way in our laboratory to investigate the potential usefulness of conjunctival epithelium to reconstruct the corneal surface in patients with limbal stem cell deficiency.

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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

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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-