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 keratoprosthetic 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 Tesque 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. Venesecion 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 mm²) 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 × 10⁴ cells/cm². 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% CO₂–95% 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.

**Clony-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

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\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 × 10⁶ cells/well. These were cocultured with mitomycin-C-inactivated 3T3 fibroblasts (2 × 10⁴ cells/cm²).17,18 The cells were incubated with AS- and FBS-supplemented culture media, as described above. The cultures were submersed 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% CO₂–95% 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.
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.
There were also no statistically significant differences between them.

Antibody omission exhibited no discernible specific immunostaining, incubated with normal mouse and goat IgG, and primary investigated immunohistochemically. Negative control sections of cultivated corneal (Fig. 3) and oral (Fig. 4) epithelium were also no statistically significant differences between the CFEs (Figs. 3A1, 3B1, 3A2, 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).

**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 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 keratin 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).

**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.

**Basement Membrane Assembly**

**Protein Expression**

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

**Electron Microscopy**

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

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

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**References**


