An Evaluation of Cultivated Corneal Limbal Epithelial Cells, Using Cell-Suspension Culture

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PURPOSE. A previous report has described an ocular surface reconstruction method involving the use of cultivated corneal epithelium derived from limbal explants. In the current study, a new culture system was developed involving in vitro propagation on amniotic membrane (AM) of epithelial cells from enzymatically dissociated limbal epithelium. The purpose of this new method is to produce a cultivated epithelial cell layer that contains stem cells and that is superior to explanted cultivated epithelium. The new cell-suspension technique was compared with the existing explant method.

METHODS. Limbal epithelial cells were dissociated from donor eyes by dispase and seeded on the denuded AM. Small pieces of limbal epithelium were also cultured on denuded AM as explant cultures. The cultivated epithelium was examined by electron microscopy and immunohistochemistry for cornea-specific keratins (K3 and K12).

RESULTS. Both cell-suspension and explant culture methods produced a healthy epithelial cell layer. The cell-suspension culture had significantly (P < 0.001) more desmosomal junctions between the explant-cultured basal cells. In addition, the intercellular spaces between the cell-suspension’s basal cells were significantly (P < 0.001) smaller than those between the explant-cultured basal cells. Both types of cultivated epithelium showed positive expression of K3 and K12 keratins. In the cell-suspension culture, expression of K3 and K12 keratins was more prominent in the superficial cells.

CONCLUSIONS. Corneal epithelial cells were successfully regenerated in vitro by a cell-suspension culture system. The suspension-cultured epithelium must include some stem cells and morphologically is significantly superior to explant-cultured epithelium. Thus, this new technique is potentially more suitable for cultivated corneal limbal epithelial transplantation.


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Supported by Grant 12557149 from the Japanese Ministry of Health and Welfare and the Japanese Ministry of Education and grants from the Kyoto Foundation for the Promotion of Medical Science; the Intramural Research Fund of the Kyoto Prefectural University of Medicine; the Alexander von Humboldt Foundation, Bonn, Germany; the EPSRC, the Wellcome Trust, United Kingdom; and the TFC Frost Trust, United Kingdom.

Submitted for publication January 17, 2002; accepted February 28, 2002.

Commercial relationships policy: N.

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The healthy ocular surface is composed of two different types of epithelial cells: corneal epithelial and conjunctival epithelial cells. Corneal epithelial cells are essential for a clear cornea and good vision. However, in severe ocular surface diseases, such as Stevens-Johnson syndrome and chemical burns, the corneal epithelial cells are sometimes totally destroyed. The damage often extends to the limbal area of the cornea, where the corneal epithelial stem cells are located.1,2 In a severely injured cornea, in which the limbal and central epithelia are both absent, the neighboring conjunctival epithelial cells invade the corneal surface, and visual acuity is severely obstructed.3,4

The most recent treatment for severe ocular surface diseases involves the transplantation of cultivated corneal epithelial cells. This latest technology for reconstructing damaged ocular surfaces in stem cell deficiencies and was first reported in 1997 by Pellegrini et al.5 After this report, the next major advance in the development of this new surgical technology was the use of human amniotic membrane (AM) as a substrate for in vitro epithelial cell culture. AM by itself has been used in ocular surface reconstruction surgery for some time,6–10 and the working mechanism of the AM on the ocular surface has been investigated.11–16 Thus, the use of the AM as a substrate for corneal epithelial cell culture was a logical progression.17,18 Our group first cultivated rabbit limbal epithelial cells on AM in vitro and then, after transplantation onto the rabbit ocular surface, confirmed the viability of the transplanted cultivated epithelium in vivo.17 Next, we showed that a denuded AM substrate (without amniotic epithelial cells) is better for corneal epithelial cell culture than cellular AM (with amniotic epithelial cells).20 In the current study, we used a method of cultivated corneal limbal epithelial transplantation with a denuded AM carrier for clinical use in cases of severe stem cell deficiencies,21–22 including acute-phase Stevens-Johnson syndrome, which is considered to be a contraindication for corneal transplantation.

Several encouraging results have been reported on the transplantation of cultivated corneal epithelial cells.17–22 However, some of the challenges of this new procedure are now becoming clear.23 One of the biggest problems is the difficulty in identifying stem cells within the cultivated epithelium. The existence of stem cells is linked to specific keratin expression,1 but, to date, there are no reliable markers that identify only stem cells. It is important to include stem cells in the cultivated corneal epithelial sheet, because they influence the longevity of the cultivated corneal epithelium. Currently, to our knowledge, every investigator who cultures the corneal epithelial cells on an AM carrier for ocular surface reconstruction produces cultivated corneal epithelial cells from corneal limbal explants. The benefits of using explants are that they are easy to prepare and there is no danger of damaging the donor’s corneal epithelium through enzyme treatment. However, it has been suggested that limbal stem cells do not readily migrate from the limbal explants onto the AM carrier. Because surgeons usually remove the explants themselves from the cultivated corneal sheet before the transplantation, to obtain a smooth reconstructed corneal surface, it is possible that the
transplanted corneal sheet may not contain many limbal stem cells. With cell-suspension culture, the cultivated corneal epithelial sheet would be generated from dissociated single cells that would include limbal stem cells. Thus, the cell-suspension–cultivated epithelium should contain a significant proportion of stem cells. Using this method, it would also be possible to transplant the whole membrane, because there would be no explants to remove from the cultivated epithelial sheet.

In this article we report how we developed and evaluated a cell-suspension system for the culture of corneal epithelial cells on an AM carrier. The cultured cells were derived from single cells in suspension and dissociated from limbal rings and therefore must include limbal stem cells. We report the results of a comparison of our new cell-suspension culture system with the existing explant culture system that was performed with light, scanning, and transmission electron microscopy and immunohistochemistry for corneal epithelium–specific keratins.

**Materials and Methods**

**Preparation of AM and 3T3 Fibroblast Cells**

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human AMs were obtained at the time of Cesarean section. The membranes were washed with sterile phosphate-buffered saline (PBS) containing antibiotics (5 mL 0.3% ofloxacin) under sterile conditions, and stored at ~80°C in Dulbecco’s modified Eagle’s medium (GibcoBRL, Rockville, MD) and glycerol (Nacalai Tesque Co., Kyoto, Japan) at a ratio of 1:1 (vol/vol). Immediately before use, the AM was thawed, washed three times with sterile PBS, and cut into pieces approximately 2.5 cm². Membranes were then deprived of their amniotic epithelial cells by incubation with 0.02% EDTA (Waco Pure Chemical Industries, Osaka, Japan) at 37°C for 2 hours to lessen cellular adhesion, followed by gentle scraping with a cell scraper (Nalgé Nunc International, Naperville, IL).

As we have reported, 17–20–23 we cultured the corneal limbal stem cells by using a modified keratinocyte culture system. 24 Corneal epithelial cells were cocultured with inactivated 3T3 fibroblasts, as described previously, 24 with the modification that inactivation was achieved by treatment with mitomycin C (MMC). Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL MMC for 2 hours at 37°C under 5% CO₂, and then trypsinized and plated onto plastic dishes with a density of 2 × 10⁴ cells/cm². Denuded AMs (measuring approximately 2.5 cm²) were spread, epithelial basement membrane side up, on the bottom of culture plate inserts (Millpore Corp., Bedford, MA), and these inserts were placed in dishes containing treated 3T3 fibroblasts.

**Cell-Suspension Culture of Corneal Limbal Epithelial Cells**

Human corneal tissue supplied from Northwest Lion Eye Bank (Seattle, WA) was used for both cell-suspension and explant cultures. The transplantation of the corneal epithelial cells cultivated on AM was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. For cell-suspension culture, the whole limbal ring was cut into two to three pieces, and these were incubated at 37°C for 1 hour with 1.2 IU dispase, as described by Gibson and Grill. 25 We modified the reported method slightly, with a change in duration of the incubation. The corneal limbal epithelium, including the stem cells, was suspended in 3 mL medium (5–10 × 10⁴ cells/mL medium), seeded onto three pieces of denuded AM spread on the bottom of culture inserts, and cocultured with MMC-inactivated 3T3 fibroblasts. The culture was submerged in the medium for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 2 weeks to promote corneal epithelial differentiation. The culture medium used was Dulbecco’s modified Eagle’s medium and Ham’s F12 (1:1 mixture) and included fetal bovine serum (10%), insulin (5 μg/mL), cholera toxin (0.1 nmol/L), epidermal growth factor (10 ng/mL), and penicillin-streptomycin (50 IU/mL). Cultures were incubated at 37°C in 5%CO₂-95% air for up to 28 days, with the medium changed every 2 days.

Just after the dispase treatment, the residual corneal rings were prepared for scanning electron microscopic examination (as described later) to confirm that all the corneal stem cells had been removed from the limbal rings.

**Explant Culture of Corneal Limbal Epithelial Cells**

The explant culture was made using a method that we have reported. 20 Briefly, the limbal tissue was cut into 2-mm², 100-μm-thick explants. Three pieces of the explant were placed directly, epithelium side down, on a portion of denuded AM spread on a culture plate insert. The culture was submerged into the medium described earlier for 2 weeks and then exposed to air by lowering the medium level (airlifting) for 2 weeks. The explants were left in the culture dish for the whole duration of the incubation.

**Light Microscopy**

The samples were fixed with 10% buffered neutral formaldehyde, routinely processed, and examined after hematoxylin and eosin staining.

**Scanning Electron Microscopy**

The samples were fixed in 4% glutaraldehyde in PBS. They were washed in PBS for 15 minutes and postfixed in 2% osmium tetroxide for 2 hours. They were washed again in PBS before being passed through an alcohol series. After two 20-minute changes of 100% ethanol, the samples were then transferred to hexamethyldisilane for 10 minutes and air dried. The samples were then mounted on aluminum specimen stubs and sputter coated with gold before being examined on a scanning electron microscope (model JSM 5600; JEOL, London, UK).

**Transmission Electron Microscopy**

The samples were fixed in 4% glutaraldehyde in PBS. They were washed in PBS for 15 minutes and then postfixed in 2% osmium tetroxide for 2 hours. They were washed again in PBS before being passed through an alcohol series and embedded in Araldite resin (Agar Scientific, Ltd., Stansted, UK). Ultrathin sections were cut on a microtome (Ultracut E; Reichert Jung, Vienna, Austria), collected on bare copper grids, and stained with aqueous uranyl acetate, phosphotungstic acid, and lead citrate before examination with a transmission electron microscope (JEOL 1010; JEOL).

**Quantitative Analysis of Explant and Cell-Suspension Cultures**

A quantitative comparison of intercellular spaces and desmosome numbers in neighboring basal epithelial cells was performed. Images of the explant- and cell-suspension–cultured epithelial cells were digitized with a scanner (Perfection 1240U; Epson Seiko Corp., Nagano, Japan). Regions of the interface of adjacent basal cells from both explant (n = 14) and cell-suspension (n = 14) cultures were selected at random by an individual who was not a participant in the project. Each selected region corresponded to a 3-μm-long interface between adjacent cells. The cellular area (the area of extracellular space between the adjacent cells) was determined on computer (Optimas 6 Image Analysis Software; Optimas, Bothell, WA). The number of desmosomes between adjacent cells within the same randomly selected regions was counted manually. Statistical analysis of the differences in the intercellular volume and in the number of desmosomes was performed with the Mann-Whitney rank sum test (Sigma Stat software; SPSS Science, Chicago, IL).
Immunohistochemistry for Keratin 3 and Keratin 12

We performed indirect immunohistochemical studies of the corneal-specific keratin (K3 and K12), by using our method as described.26 Cryostat sections (7μm thick) were placed on gelatin-coated slides, air-dried, and dehydrated in PBS at room temperature for 15 minutes. Normal human corneal tissue was used as the positive control. The sections were incubated with 1% bovine serum albumin (BSA) at room temperature for 30 minutes to block nonspecific binding. The sections were then incubated at room temperature for 1 hour with the primary antibody and washed three times in PBS containing 0.15% Triton X-100 (PBST) for 15 minutes. Anti-keratin 3 antibodies (AE3, mouse monoclonal) were purchased commercially (Progen Biotechnik, Heidelberg, Germany), and anti-keratin 12 (J7, rabbit polyclonal) antibodies were kindly provided by Michelle Kurpakus (Department of Anatomy and Cell Biology, Wayne State University, Detroit, MI).27 In negative control experiments, the primary antibody was omitted. After staining with the primary antibodies, the sections were incubated at room temperature for 1 hour with suitable secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) and FITC-conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, CA). After they were washed several times with PBS, the sections were coveredslipped with anti-fading mounting medium containing propidium iodide (Vectashield; Vector Laboratories), and the slides examined by confocal microscopy (Fluoview; Olympus, Tokyo, Japan).

RESULTS

Light Microscopy

Both culture methods produced five to six layers of well-stratified epithelium (Figs. 1A, 1B). The cells appeared healthy and were differentiated into basal column-shaped cells, suprabasal cuboid wing cells, and flat squamous superficial cells. In both cultures, the epithelium attached well to the denuded AM.

Scanning Electron Microscopy

Examination of the residual corneal rings after dispase treatment confirmed that all the corneal limbal cells, including corneal stem cells, had been removed from the limbal rings. Figure 2 shows that only basement membrane with an irregular surface was visible.

Examination of the apical surface of the cells showed a continuous layer of flat polygonal epithelial cells in both the explant and the cell-suspension cultures (Figs. 3A, 3D). The cells averaged approximately 50 to 60 μm in diameter and appeared similar to in vivo human corneal epithelial cells. Desquamating cells were observed on the surface of both the explant and the cell-suspension cultures. In the cell-suspension culture, the junctions between the superficial cells appeared slightly more prominent and more tightly opposed than in the explant cultures (Figs. 3B, 3E). The surface of both the explant- and the cell-suspension-cultured cells were covered with numerous small microvilli (Figs. 3C, 3F).

Transmission Electron Microscopy

Examination at low magnification confirmed the light microscopy findings. Both culture techniques produced five to six layers of well-stratified epithelium that were clearly differentiated into basal column-shaped cells, suprabasal cuboid wing cells, and flat squamous superficial cells. The superficial and suprabasal cell layers were similar in appearance and had similar numbers of desmosomal junctions in both explant- and cell-suspension–cultured material. These features are shown in Figure 4A for the explant-cultured cells and in Figure 4D for the cell-suspension–cultured cells. However, there were also considerable structural differences between the cell-suspension–cultured and explant-cultured epithelium. These differences were seen in the basal regions of the epithelial cell layer, the most obvious of which were the much larger intercellular areas between the cells in the basal layer present in the explant-cultured epithelium (Fig. 4A). In contrast, the cell-suspension–cultured epithelium had very small intercellular spaces between the basal cells (Fig. 4D). Both types of culture methods produced a substantial basement membrane layer (Figs. 4B, 4E), with evidence of hemidesmosome–like attachments of the basal cells. However, the basal surface of the cell-suspension–cultured epithelium (Fig. 4E) was much flatter and less undulating than the basal surface of the explant-cultured epithelium (Fig. 4B), which showed deep interdigitations into the basement material. Finally, the cell-suspension culture epithelium...
had many more desmosomal junctions between the basal epithelium (Fig. 4F) than were present between the basal cells of the explant-cultured epithelium (Fig. 4C).

Quantitative Analysis of Explant and Cell-Suspension Cultures

There was a highly significant ($P < 0.001$) difference between explant and cell-suspension cultures in the area of the intercellular spaces of neighboring basal epithelial cells. Explant cultures had an average intercellular area of 5.42 ± 2.05 $\mu$m$^2$ (SD; $n = 14$; Fig. 5, top), whereas cell-suspension cultures had average intercellular area of 0.28 ± 0.18 $\mu$m$^2$ ($n = 14$). There was also a significant difference ($P < 0.001$) between explant and cell-suspension cultures in the number of desmosomes between neighboring basal epithelial cells. Explant cultures had a desmosome average of 0.29 ± 0.59 (SD; $n = 14$; Fig. 5, bottom), whereas cell-suspension cultures had a desmosome average of 3.57 ± 0.82 ($n = 14$). The averages for intercellular area and number of desmosomes are given for a 3-$\mu$m-long interface between adjacent cells.

Immunohistochemistry for Keratin 3 and Keratin 12

Expression of cornea-specific keratin markers (K3 and K12) was examined in both explant- and cell-suspension-cultured epithelial cells by immunohistochemistry. We observed positive staining against K3 and K12 in both types of cultured epithelial cells (Fig. 6), and in cell-suspension culture, K3 and K12 expression was more prominent in the superficial cells.

Discussion

Cell-suspension culture, which means the culture from single cells, was first developed for clonal growth assays$^{28}$ and for transplantation.$^{29,30}$ Lindberg et al.$^{29}$ in a pioneering study, cultivated an undifferentiated epithelium a few layers thick from single cells, and then produced stratified human corneal epithelium by transplanting this cultivated epithelium subcutaneously into the nude mouse. It is likely that this xenograft transplantation supplied the substrate and also worked as a physiological dressing for the delicate epithelium. Although a bioengineered three-dimensional cornea is being developed from cells derived from cell-suspension culture for drug toxicity testing,$^{31}$ currently, there are no clinical applications of the transplantation of cultivated corneal epithelial cells derived from cell-suspensions using an AM carrier.

The purpose of this study was to compare our cell-suspension culture technique with the existing explant culture technique. Although the explant culture technique has been extremely successful,$^{21,22}$ questions regarding the very-long-term...
viability of the transplanted epithelium remain. In theory, our new method should produce superior results, because the cell-suspension technique seeds limbal stem cells directly onto the AM substrate. Because no one to date has identified a stem cell marker, we could not identify stem cells directly. Therefore, we used light, scanning, and transmission electron microscopy, to perform a detailed comparison of the morphology and ultrastructure of the epithelial cell layers, and immunohistochemistry, to determine the expression of the cornea-specific K3 and K12 markers.

We modified the method of Gipson and Grill for collecting the limbal epithelium, which includes the most basal cell layers. In the original paper, they treated fresh rabbit corneal limbal tissue for 3 hours with 1.2 U dispase to collect the whole layer of the limbal epithelium. We changed the duration for dispase reaction to 1 hour, because our corneal limbal tissue came from an eye bank in the United States, and these eyes are kept in storage medium (Optisol; Chiron Vision, Irvine, CA) for 5 to 6 days after enucleation. Consequently, the attachment between the basal layer of the epithelium and the basement membrane is less strong. Our SEM examination of the limbal ring after dispase digestion clearly shows that all the limbal epithelial cells were removed by this treatment, and therefore the stem cells presumably were seeded onto the AM.

Our results from this study show that both cell-suspension and explant culture methods produced a five- to six-cell-thick, well-stratified and differentiated epithelial cell layer. These layers contained healthy cells with normal cell junctions and produced basement membrane material. On the apical surface some cells appeared to be undergoing the process of desquamation. However, there are differences between the two types of cultured epithelia. Examination of the apical surfaces of the cultured epithelium by scanning electron microscopy showed that the cell-suspension-cultured epithelial cells had more tightly opposed and more distinct cell junctions (Fig. 3E) than explant-cultured epithelial cells (Fig. 3B) and thus would be expected to provide a superior barrier. Transmission electron microscopy also showed that the cell-suspension-cultured epithelial cells had significantly more desmosomal junctions (Fig. 5, bottom) and significantly smaller (Fig. 5, top) intercellular spaces between the basal cells. Thus, the cell-suspension-cultured epithelium would have greater structural integrity and mechanical strength than the explant-cultured epithelium. We speculate that in the cell-suspension-cultured epithelium a higher proportion of stem cells in the basal layer results in higher proliferation rates in the basal layer, which in turn results in the basal cells becoming more closely packed together and reduces the intercellular area. Higher rates of cell
proliferation may account for the more tightly opposed cell junctions and smaller intercellular spaces present between the cells in the cell-suspension–cultured epithelium. Regarding corneal epithelium–specific differentiation, the more diffuse expression of K3 and K12 occurred in explant-cultured epithelium. In contrast, the expression of K3 and K12 in the superficial epithelium of cell-suspension–cultured epithelium was more prominent the basal epithelium, indicating that the cell-suspension system promotes normal differentiation of cultivated epithelium.

Thus, morphologically, the cell-suspension–cultured epithelium is significantly better than the explant-cultured epithelium, is closer in appearance to a normal epithelial cell layer, and is therefore potentially more suitable for transplantation. Also, although we cannot directly confirm that stem cells are present in the cell-suspension–cultured epithelium, our method of seeding limbal stem cells onto the AM means that some stem cells must be present—an assumption that cannot be made about explant cultures. Examination of the limbal ring by electron microscopy (Fig. 2) after treatment by dispase

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**Figure 5.** Top: differences in intercellular spaces between adjacent basal epithelial cells that were cultured with the explant technique (average, $5.42 \pm 2.05 \mu m^2; n = 14$) and the cell-suspension technique (average, $0.28 \pm 0.18 \mu m^2; n = 14$). Bottom: difference in number of desmosomes between adjacent basal epithelial cells that were cultured with the explant technique (average, $0.29 \pm 0.59; n = 14$) and those cultured with the cell-suspension technique (average, $3.57 \pm 0.82; n = 14$). The averages for intercellular spaces and number of desmosomes were calculated for a 3-\(\mu m\)-long interface between adjacent cells. Both differences in intercellular spaces and number of desmosomes were statistically significant ($P < 0.001$).
confirmed that all stem cells had been transferred from the limbal ring into cell-suspension culture.

As a result of this evidence we have already transplanted the cultivated epithelium made by the cell-suspension system onto seven eyes with severe ocular surface diseases, such as Stevens-Johnson syndrome and chemical burns. Four of these seven eyes had received transplants of explant-cultured epithelium and had subsequently shown conjunctival invasion into the cultivated allocorneal epithelial transplant. Fortunately, in cases in which the initially transplanted cultivated epithelium becomes compromised, it is relatively simple to repeat the transplantation process with new cultivated epithelium on AM. All seven eyes showed perfect coverage with transplanted cell-suspension–cultured corneal epithelium 48 hours after transplantation. Slit lamp examination after fluorescein staining, 48 hours after the transplantation, showed that the fluorescein permeability of the transplants was very low, similar to that in healthy in vivo corneas. Although we have not conducted a quantitative clinical comparison of the barrier function between explant- and cell-suspension–cultured transplants (for example, by fluorophotometry), our clinical observations with fluorescein staining suggest that the barrier function of the cell-suspension cultures is better than that of the explant cultures. The clinical superiority of cell-suspension–cultured epithelium may be related to its tightly opposed cell junctions (Fig. 3E), smaller intercellular spaces, and increased numbers of desmosomes (Fig. 5). These features would be expected to improve the mechanical strength and barrier function of the cell sheet. Thus, it would be better able to remain intact and retain its barrier function when exposed to the stresses and minor abrasions associated with transplantation and the ocular surface environment.

As for the function of the cultivated corneal epithelium, although we have not examined the mucin expression on the cultivated corneal epithelium, we have some preliminary data from our ongoing study that indicate cell-suspension–cultured epithelium expresses several cornea-specific proteins, such as apolipoprotein J (clusterin). This is known to be produced specifically in the corneal epithelium, and these results suggest the cultivated epithelium performs similarly to normal corneal epithelium.

At this stage, it is difficult to compare the viability of the transplanted epithelium cultured by explant and cell-suspension methods, because in both methods, the survival rate of the transplanted epithelium was incredibly good (100% of transplanted epithelium survived in results confirmed at 48 hours after transplantation). However, we think the long-term survival of these transplanted epithelia reflects the fact that the cell-suspension–cultured epithelium contains some stem cells, because they have been removed from limbal rings (Fig. 2), whereas it has been suggested that the explant-cultured epithelium may contain only transiently amplified cells. Currently, we are carefully monitoring the transplanted cell-suspension–cultivated corneal epithelium to determine whether the lon-

**Figure 6.** Immunohistochemical staining of K3 and K12 in explant- and cell-suspension–cultured epithelial cells. (A) K3 and (B) K12 expression in the explant culture. (C) Negative control for K3 expression. (D) K3 and (E) K12 expression in the cell-suspension culture. (F) Negative control for K12 expression. Both explant- and cell-suspension–cultured epithelial cells showed positive staining for K3 and K12. Also, in the cell-suspension culture, K3 and K12 expression was more prominent in the superficial cells. Scale bar, 100 μm.
The gravit of the cell-suspension culture is greater than that of the explant culture. In conclusion, we believe our new cell-suspension culture system is a significant contribution toward the improvement of cultivated corneal epithelial cell transplantation for ocular surface reconstruction.

References


