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Short communication

The efficient fabrication of corneal epithelial cell sheets by controlling oxygen concentration

Ryota Nakajima, Shizu Takeda*

Central Research Laboratory, Hitachi Ltd., 2520 Hatoyama, Saitama 350-0395, Japan

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ABSTRACT

We have developed a novel method to accelerate the fabrication of epithelial cell sheets by controlling oxygen concentration. Rabbit limbal epithelial cells were proliferated efficiently under hypoxia (2% O_2) in comparison to those proliferated under normoxia (20% O_2), but were not stratified completely under 2% O_2. In contrast, corneal limbal epithelial cells cultured under hypoxia were stratified by re-oxygenation after reaching confluence. Histological and immunofluorescence analyses and colony-forming assays showed that it was possible to fabricate the corneal epithelial cell sheets efficiently by controlling the oxygen concentration. These results indicate that this novel method can be a cost-effective tool for fabricating stratified epithelial cell sheets for corneal regenerative medicine.

Tissue-engineered corneal epithelial and oral mucosal epithelial cell sheets have been utilized in clinical applications to treat ocular surface diseases such as chemical and thermal burns or Stevens-Johnson syndrome, which severely damages the limbus (Nishida et al., 2004; Rama et al., 2010). Conventional methods of fabricating the stratified epithelial cell sheets require several weeks for *ex vivo* expansion of epithelial cells including stem cells and stratification. In clinical applications, the processes of fabricating stratified cell sheets in Good Manufacturing Practice (GMP) compliant facilities place an enormous burden on the experts who fabricate them (Takagi et al., 2011). Therefore, efficient methods for fabricating the cell sheets need to be developed. If the manufacturing period is shortened, the production of tissue-engineered cell sheets will become cost-effective.

Recent reports showed that low oxygen concentration enhances the proliferation of various stem cells (Mohyeldin et al., 2010) including limbal epithelial cells (Miyashita et al., 2007; O'Callaghan et al., 2011). On the other hand, we previously discussed that the oxygen concentration may affect the stratification of corneal epithelial cell sheets (Nakajima et al., 2012). Based on these investigations, we have developed a novel method in which limbal epithelial cells are cultured under hypoxia in the proliferation stage and then cultured under normoxia during stratification to shorten the fabrication period of the cell sheets. Here, we show that corneal epithelial cell sheets can be fabricated efficiently with the novel method using primary rabbit limbal epithelial cells.

Rabbit limbal tissues were isolated from Japanese white house rabbit ocular globes purchased from Japan Lamb Ltd. Limbal tissues were incubated with 200 U/mL of dispase II (Sanko Junyaku, Tokyo, Japan) at 37 °C for 1 h. The separated epithelial layer was treated with 0.25% trypsin–0.01 mM EDTA solution (Nacalai Tesque), and resuspended primary cells were plated on cell-culture inserts (six-well, pore size: 0.4 μm; BD Biosciences, San Jose CA) at a density of 2×10^4 cells/cm^2 in a keratinocyte culture medium as previously described (Nakajima et al., 2012). Cell-culture inserts have microporous membrane to supply nutrition from the apical and basal sides of the inserts, with or without feeder cells attached to the multi-well plates (Hayashida et al., 2005; Nishida et al., 2004). To prepare lethally treated feeder cells, NIH/3T3 cells were incubated with 10 μg/mL mitomycin C (Roche Diagnostics, Basel, Switzerland) at 37 °C for 2 h and then cells were plated in six-well plates at a density of 2×10^4 cells/cm^2. The rabbit corneal limbal epithelial cells were cultured in normoxic conditions (condition 1: referred to as O_2 20%) using a CO_2 incubator, at 37 °C in a humidified 5% CO_2 environment as a control. The cells were also cultured in hypoxic conditions (condition 2: named O_2 2%) using multi-gas incubators (APM–30D, Astec, Fukuoka, Japan) with N_2 gas at 37 °C in a humidified 5% CO_2 environment. When the cells proliferated to reach confluence and had a cobble stone-like morphology, the culture dishes were transferred to a CO_2 incubator to re-oxygenate the cells for 4 days (condition 3: named O_2 2 → 20%). The cells were cultured for 12–14 days. The cultured epithelial cells were observed

* Corresponding author. Tel.: +81 49 296 6111; fax: +81 49 296 6006.
E-mail address: shizu.takeda.me@hitachi.com (S. Takeda).

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with a phase contrast microscope (Axiovert 200; Carl Zeiss, Jena, Germany). After being cultured, the epithelial cell sheets were harvested with 200 U/mL Dispase (Sanko Junyaku) at 37 °C for 15 min in a feeder layer. Harvested cell sheets were treated with trypsin–EDTA solution, and the cell number and cell viability of resuspended cells, which were stained with trypan-blue, were counted with an automated cell counter (TC10; Bio-Rad laboratories, Hercules, CA). Cultivation was performed in duplicate for three independent groups of ocular globes ($n = 3$). Statistical analyses were performed by one-way ANOVA with *post hoc* Tukey analysis.

For DNA quantitation, rabbit limbal epithelial cells were cultured on cell-culture inserts in O_2 20% and O_2 2% for 4, 6, or 8 days independently. After being cultured, cells were washed with phosphate-buffered saline (PBS). DNA was extracted from the cultured cells using a DNA quantity kit (Primary cell Co., Ltd., Hokkaido, Japan) and the cells were stained with Hoechst 33258. The fluorescence intensity of the DNA was measured using a microplate reader (SH-8000; Corona Co., Ltd., Ibaragi, Japan). Cultivation was performed in duplicate for three independent groups of ocular globes ($n = 3$). Statistical analyses were performed by Student's *t*-test.

Hematoxylin and eosin (H&E) staining was performed with the cell sheets to examine the degree of stratification of epithelial cells. The epithelial cell sheets were embedded in a Tissue-Tek OCT compound (Sakura Seiki, Tokyo, Japan), and processed into 10-μm-thick frozen sections on cryofilm (Leica Microsystems, Tokyo, Japan). After being dried for 30 min at room temperature, the tissues were fixed with 4% paraformaldehyde at room temperature for 5 min. The sections were washed three times with distillated-deionized water (DDW) and then stained with H&E. For the immunofluorescence analyses, fixed sections were washed three times with PBS. After being treated with 5% horse or rabbit serum for 10 min to block nonspecific reactions, the sections were incubated with anti-pancytokeratin (Pan-CK) antibody (1:100 dilution, Ks pan 1–8; Progen, Heidelberg, Germany), anti-p63 antibody (1:50 dilution, 4A4; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytokeratin 3/2p (CK3) antibody (1:100 dilution, AE5; Santa Cruz Biotechnology) and anti-Claudin-1 antibody (A10; Santa Cruz Biotechnology) at 4 °C overnight. To observe p63 expression, the samples were permeabilized in 0.5% Triton X-100 in PBS at room temperature for 5 min before antibody reaction. The samples were washed three times with 0.05% Tween20 in PBS (PBST), and then incubated with biotin-conjugated anti-mouse or goat IgG antibody (4 μg/mL; Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After being washed three times with PBST, the samples were incubated in Fluorescein-labeled Streptavidin (1:500 dilution, Perkin Elmer and Analytical Science, Waltham, MA) at room temperature for 30 min. After incubation, the samples were washed three times with PBST and incubated with Hoechst 33258 at room temperature for 5 min. The samples were then washed with DDW and mounted with 30% glycerin and Perma-Fluor (ThermoFisher Scientific, Waltham, MA). Fluorescence images were observed with an optical microscope (AX70; Olympus). The procedures were repeated three times. The percentage of p63-positive cells in each cultured cell sheet was calculated from five independent sections.

For colony-forming assays (CFAs), harvested cell sheets were treated with trypsin–EDTA solution, and resuspended cells (2000 cells/10-cm culture dish) were cultured on mitomycin C treated 3T3 (1×10^4 cells/cm^2) with KCM for 11 days. After cultivation, cells were fixed with 3.7% formaldehyde and stained with 1% rhodamine B (Nacalai Tesque). Holoclone colonies greater than 2 mm in diameter were counted as described previously (Barrandon and Green, 1987) using Axiovision software (Carl Ziess, Oberkochen, Germany). The colony-forming efficiency (CFE) was calculated as the ratio of the number of colonies over the number of cells seeded (2000 cells). CFAs were performed in duplicate for three independent cell sheets ($n = 3$). Statistical analyses were performed by Student's *t*-test.

To confirm the hypoxic effect on the expansion of the corneal limbal epithelial cells, the cells were cultured under O_2 2% in cell-culture inserts. Under hypoxia, rabbit corneal limbal epithelial cells reached confluence and showed cobble stone-like morphology rapidly (day 8), in contrast to those under O_2 20% (day 10) (Fig. 1A). The fluorescence intensity of the DNA, which was extracted from cultured cells under O_2 20% and under O_2 2%, was respectively 2.19 ± 1.27 and 5.95 ± 0.60 at day 4, 4.64 ± 0.52 and 9.02 ± 0.41 at day 6, and 8.04 ± 0.70 and 13.48 ± 2.06 at day 8 (Fig. 1B). The signal intensity under O_2 2% was significantly higher than that under O_2 20% on each sampling day ($p < 0.05$). Although epithelial cells proliferated efficiently under hypoxia in correspondence with a previous report (Miyashita et al., 2007), corneal epithelial cells were not stratified completely under hypoxia (one to two cell layers) (Fig. 1B). When the corneal epithelial cells that were cultured in hypoxia and were then placed under O_2 20% after reaching confluence, the epithelial cells were stratified into three to six cell layers (Fig. 1C). This method was designated as an oxygen-controlled method. There were no defects in harvested cell sheets fabricated in all culture conditions (Fig. 1C). The number of cells in the cell sheets at day 12 in O_2 20%, O_2 2 → 20%, and O_2 2% were 4.5 ± 0.9 × 10^6, 5.9 ± 0.5 × 10^6, and 3.7 ± 0.5 × 10^6 cells, respectively (Fig. 1D). The number of cells in the cell sheets at day 14 in O_2 20%, O_2 2 → 20%, and O_2 2% were 5.8 ± 0.5 × 10^6, 6.6 ± 0.2 × 10^6, and 3.8 ± 0.1 × 10^6 cells, respectively (Fig. 1D). In O_2 2 → 20% at day 12, the cell number was significantly higher than that in O_2 20% at day 12 ($p < 0.05$) and was equal to that in O_2 20% at day 14 (Fig. 1D). The cell number in O_2 2% was significantly lower than that in O_2 2 → 20% at day 12 ($p < 0.01$) (Fig. 1D). These results indicate that the oxygen concentration affects the stratification of the corneal epithelial cells, and that cultivation period for fabrication of the stratified cell sheets can be shortened by the oxygen-controlled method.

To evaluate the fabricated epithelial cell sheets in O_2 20% (day 14) and in O_2 2 → 20% (day 12), the expressions of epithelial cell makers were confirmed through immunofluorescence analyses (Fig. 2A). CK family proteins, epithelial cell markers, were expressed in all cells of the cell sheets. CK3, a corneal differentiated epithelial cell marker (Schermer et al., 1986) was expressed in suprabasal epithelial cell layers. The putative epithelial stem cell marker, p63 (Pellegrini et al., 2001) was expressed in the basal cell layers in each condition. Claudin-1, an essential tight junction molecule for epithelial barrier functions (Ban et al., 2003) was partially expressed in the superficial cells in each condition. A recently reported study demonstrated that the existence of more than 3% of p63-bright holoclone forming stem cells in the graft is important for the success of long-term corneal regeneration for patients with limbal stem-cell deficiency (Rama et al., 2010). To confirm the existence of epithelial stem/progenitor cells in the cell sheets, quantitation of p63-positive cells and CFAs were performed. There were no significant differences between the percentages of p63-positive cells in O_2 20% (day 14) (47.6 ± 4.6%) and in O_2 2 → 20% (day 12) (42.7 ± 8.0%). The CFEs of the fabricated cell sheets were not significantly different in O_2 20% (day 14) (4.2 ± 0.7%) and in O_2 2 → 20% (day 12) (4.4 ± 1.0%) (Fig. 2B). The cell viability of the cultured cell sheets in O_2 20% (day 14) and in O_2 2 → 20% (day 12) was 97.7 ± 0.8% and 96.8 ± 1.2%, respectively.

In the present study, we reported the novel method for efficient fabrication of corneal epithelial cell sheets. O'Callaghan et al. (2011)