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Fabrication of corneal epithelial cell sheets maintaining colony-forming cells without feeder cells by oxygen-controlled method

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ABSTRACT

The use of murine 3T3 feeder cells needs to be avoided when fabricating corneal epithelial cell sheets for use in treating ocular surface diseases. However, the expression level of the epithelial stem/progenitor cell marker, p63, is down-regulated in feeder-free culture systems. In this study, in order to fabricate corneal epithelial cell sheets that maintain colony-forming cells without using any feeder cells, we investigated the use of an oxygen-controlled method that was developed previously to fabricate cell sheets efficiently. Rabbit limbal epithelial cells were cultured under hypoxia (1–10% O_2) and under normoxia during stratification after reaching confluence. Multilayered corneal epithelial cell sheets were fabricated using an oxygen-controlled method, and immunofluorescence analysis showed that cytokeratin 3 and p63 was expressed in appropriate localization in the cell sheets. The colony-forming efficiency of the cell sheets fabricated by the oxygen-controlled method without feeder cells was significantly higher than that of cell sheets fabricated under 20% O_2 without feeder cells. These results indicate that the oxygen-controlled method has the potential to achieve a feeder-free culture system for fabricating corneal epithelial cell sheets for corneal regeneration.

1. Introduction

Tissue-engineered corneal epithelial and oral mucosal epithelial cell sheets have been used to treat ocular surface conditions such as chemical and thermal burns or diseases such as Stevens-Johnson syndrome, which severely damage the limbus (Nishida et al., 2004; Pellegrini et al., 1997; Rama et al., 2001; Tsai et al., 2000). Conventionally, murine 3T3 feeder cells have been utilized for ex vivo expansion of epithelial cells including corneal epithelial cells (Pellegrini et al., 1997; Rheinwald and Green, 1975). Previous reports have indicated that the secreted soluble factors from feeder cells or extracellular matrix molecules affect the proliferation of epithelial cells and the maintenance of epithelial stem and progenitor cells (Barreca et al., 1992; Conconi et al., 1996; Florin et al., 2005; Maas-Szabowski et al., 1999; Szabowski et al., 2000; Takagi et al., 2012).

In clinical applications, however, the use of murine 3T3 feeder cells is drawback of conventional culture conditions. The US Food and Drug Administration (FDA) has classified regenerated tissues using 3T3 feeder cells as xenografts. Moreover, Martin et al. reported that the human embryonic stem cells incorporate immunogenic non-human sialic acids under conventional conditions using 3T3 feeder cells (Martin et al., 2005). Several groups reported recently that murine 3T3 feeder cells could be replaced with human adipose tissue-derived or bone marrow-derived mesenchymal stem cells, or with human dermal fibroblasts to fabricate stratified epithelial cell sheets (Oie et al., 2010; Omoto et al., 2009; Sugiyama et al., 2008). However, human tissue-derived feeder cells are isolated invasively from patients by biopsy. Moreover, clinically applied feeder cells need to be managed under a strict Standard Operating Procedure (SOP) following Good Manufacturing Practice (GMP) guidelines. Thus, a feeder-free culture system is desirable for the fabrication of epithelial cell sheets.

Murakami et al. (2006) demonstrated that the use of culture inserts allows fabrication of stratified epithelial cell sheets without feeder cells. However, epithelial stem/progenitor cell marker p63-expressing cells in the oral mucosal epithelial cell sheets were significantly decreased in the feeder-free culture system (Murakami et al., 2006). In another study supporting these findings, 3T3 feeder cells were able to maintain the expression of p63 and ABCG2 in the limbal epithelial cells (Balasubramanian et al., 2008). Recently, Rama et al. (2010) reported that the existence of more

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than 3% of p63-bright holoclone-forming epithelial cells in the graft is important for the success of long-term corneal regeneration for patients with burn-related corneal destruction associated with limbal stem-cell deficiency. Therefore, tissue-engineering technologies that can replace the effect of feeder cells need to be developed to fabricate epithelial cell sheets that maintain colony-forming cells.

Although mammalian cells including limbal epithelial cells are often cultured in ambient oxygen concentration (20–21%), many types of stem cells exist in hypoxic conditions ranging from 1 to 9% O_2 (Mohyeldin et al., 2010). Recent reports have indicated that the physiological oxygen condition promotes proliferation of various stem cells including epithelial stem cells, maintains the undifferentiated state of stem cells, and inhibits differentiation of stem cells to terminally differentiated cells (Ezashi et al., 2005; Miyashita et al., 2007; Mohyeldin et al., 2010; O'Callaghan et al., 2011; Xia et al., 2012). Previously, we developed an oxygen-controlled method for efficient fabrication of corneal epithelial cell sheets (Nakajima and Takeda, 2013). This method is based on the behavior of limbal epithelial cells under hypoxia (Miyashita et al., 2007) and the demand of oxygen supplied for stratification of the epithelial cells (Nakajima et al., 2012; Ngo et al., 2007).

In the present study, we applied the oxygen-controlled method to fabricate corneal epithelial cell sheets that maintain colony-forming cells without the use of feeder cells, that is; rabbit limbal epithelial cells were cultured without murine 3T3 feeder cells in hypoxic conditions (1–10% O_2) during expansion to confluence and cultured in normaxic conditions (20% O_2) during stratification. The colony-forming efficiency, cell growth, and degree of stratification were analyzed to verify the effectiveness of this novel method for fabricating stratified corneal epithelial cell sheets.

2. Materials and methods

2.1. Cultivation of rabbit limbal epithelial cells

Rabbit limbal tissues were isolated from Japanese white house rabbit ocular globes purchased from Japan Lamb Ltd. Limbal tissues (2–3 cm diameters) 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, Kyoto, Japan) for 15 min, and resuspended cells were plated on cell-culture inserts (6-well or 12-well, pore size: 0.4 μm; BD Biosciences, San Jose, CA) at a density of 1×10^4 cells/cm^2 with or without feeder cells in a keratinocyte culture medium (KCM), which was composed of Dulbecco's Modified Eagle Medium (DMEM)/F12 (3:1) supplemented with 5% fetal bovine serum (BioWest, Nuaillé, France), antibiotics and antimycotics (Nacalai Tesque), 0.5% Insulin–Transferrin–Selenium–G supplement (Life Technologies, Carlsbad, CA), 1 nM cholera toxin (Calbiochem, Darmstadt, Germany), 2 nM triiodothyronine (MP Biomedicals, Aurora, OH), 0.4 μg/mL hydrocortisone succinate (Wako, Tokyo, Japan), and 10 ng/mL EGF (PeproTech, Rocky Hill, NJ). Cell-culture inserts have micro-porous membrane to supply nutrition from the apical and basal sides of the inserts, with or without feeder cells attached to the multi-well plates. To prepare lethally treated feeder cells, NIH/3T3 cells were incubated with 10 μg/mL mitomycin C (Roche Diagnostics, Basel, Switzerland) (MMC-3T3) at 37 °C for 2 h and then cells were plated in 6-well or 12-well plates at a density of 2×10^4 cells/cm^2. The rabbit limbal epithelial cells were cultured in normoxic conditions (20% O_2) using a conventional CO_2 incubator at 37 °C in a humidified 5% CO_2 environment as a control. In other conditions, the cells were cultured in four different oxygen tension conditions, 1, 2, 5, and 10% O_2 using multi-gas incubators (APM–30D; Astec, Fukuoka, Japan) with N_2 gas, each at 37 °C in a humidified 5% CO_2 environment. When the cells were proliferated to reach confluence and indicated a cobble stone-like morphology, the culture dishes were transferred to a CO_2 incubator to re-oxygenate the cells for 5 days. The cells were cultured for 14–17 days. Culture conditions are summarized in Table 1. The cultured epithelial cells were observed with a phase contrast microscope, and microphotographs were taken at 100-fold magnification (Axiovert 200; Carl Zeiss, Jena, Germany).

2.2. Colony forming assay

Primary rabbit limbal epithelial cells were cultured on MMC-3T3 in 10-cm tissue culture polystyrene dish (TCPS) (AGC Techno Glass, Tokyo, Japan) (17 cells/cm^2) with KCM for 10 days, and were cultured in 10-cm TCPS (35 cells/cm^2) without feeder cells for 14 days in five different oxygen tension conditions, 1, 2, 5, 10, and 20% O_2 using multi-gas incubators (APM–30D; Astec) with N_2 gas, each at 37 °C in a humidified 5% CO_2 environment. After cultivation, cells were fixed with 3.7% formaldehyde and stained with 1% rhodamine B (Nacalai Tesque), and colony number and colony size were calculated. Colony-forming assays (CFAs) were performed in duplicate for three independent groups of ocular globes.

For the secondary CFAs (2nd CFAs), harvested cell sheets were treated with trypsin–EDTA solution, and resuspended cells were cultured on MMC-3T3 in TCPS (35 cells/cm^2) with KCM for 11 days in normoxic conditions (20% O_2) using a conventional CO_2 incubator, at 37 °C in a humidified 5% CO_2 environment. After cultivation, the 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). The colony-forming efficiency (CFE) was calculated as the ratio of the number of colonies over the number of cells seeded (2000 cells). The 2nd CFAs were performed in triplicate for four independent cell sheets.

2.3. Cell sheet desquamation and total cell number

After being cultured, the epithelial cell sheets were incubated with 200 U/mL Dispase (Sanko Junyaku) at 37 °C for 15 min in a feeder layer. Then the epithelial cell sheets were harvested with tweezers. The harvested epithelial cell sheets were incubated with trypsin–EDTA solution at 37 °C for 10 min to obtain a single-cell suspension. After centrifugation, cells were resuspended in phosphate-buffered saline (PBS), and stained with 0.4% trypan blue. The number of cells was counted with an automated cell counter (TC10; Bio-Rad Laboratories, Hercules, CA).

2.4. Immunofluorescence

The epithelial cell sheets were embedded in Tissue-Tek OCT Compound (Sakura Seiki, Tokyo, Japan), and processed into 10-μm-thick frozen sections on cryofilm (Leica Microsystems, Tokyo,

Table 1
Culture conditions for fabrication of corneal epithelial cell sheets.

No.	Culture condition	Oxygen concentration	
		Proliferation of limbal epithelial cells	Stratification (5 days)
1	20% O_2	20%	20%
2	1 → 20% O_2	1%	20%
3	2 → 20% O_2	2%	20%
4	5 → 20% O_2	5%	20%
5	10 → 20% O_2	10%	20%