·Basic Research·

Effect of hypoxia on the proliferation of murine cornea limbal epithelial progenitor cells *in vitro*

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Abstract

• AIM: To investigate the effect of hypoxia on the proliferation of mouse corneal epithelial cells *in vitro*.

• METHODS:Mouse corneal epithelial cells(MCEs) were cultured in normoxia (210mL/L O_2 and 50mL/L CO_2) and hypoxia (20mL/L O_2 and 50mL/L CO_2), respectively. Colony forming efficiency (CFE) and cell proliferation were determined. The expression of corneal epithelial progenitor cell marker p63 and K19 was investigated by immunostaining.

• RESULTS: Normoxic colonies were smaller compared with colonies formed in hypoxia. CFE was (12.50 ± 1.50)% in hypoxic cultures, which was similar compared with normoxia cultures [(11.13± 1.86)%, P>0.05)]. Cell proliferation was enhanced in hypoxia. Progenitor markers p63 and K19 were expressed in most cells under both normoxic and hypoxic conditions.

• CONCLUSION: Murine limbal epithelial progenitor cells can be efficiently expanded in hypoxic conditions.

• KEYWORDS: hypoxia; cornea; epithelium; cell culture

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INTRODUCTION

Cultivated limbal epithelial cell sheets are used clinically for reconstructing the ocular surface in blinding diseases that destroy the corneal epithelial stem cell niche located in the limbus. The high yield of cells with high proliferative potential is one of the keys of successful limbal epithelial cell culture. Recently, the use of hypoxic incubation was reported to enhance progenitor cells in the bone marrow, neural cells, and epidermal keratinocytes. We therefore hypothesized that hypoxia can also be used to induce immature cells to expand from the murine limbus and enhance the expansion of epithelial progenitor cells *in vitro*.

MATERIALS AND METHODS

Materials C57BL/6 mice (CLER, Tokyo, Japan), aged 8-10 weeks, were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eye globes were enucleated from the mice with forceps after death, washed profusely in PBS. Eyes from each animal were kept separate throughout the culture procedure. Corneal buttons including the limbus were cut from the eye and cleaned of extraneous tissue (e.g. iris, ciliary body, *etc*.). Primary cell culture was performed using explants culture method similar to Hazlett *et al* Briefly, the button was cut in half and each explant with epithelium side up was plated flat on 6-well plate, one piece per well, at 37℃, under 95% humidity and 50mL/L CO_2 using an N_2/CO_2 multigas incubator (APM.-30D; Astec, Fukuoka, Japan). After approximately 10 minutes to allow for attachment of the explant, serum-free low-Ca^{2+} medium (defined keratinocyte serum-free medium, KSFM; Invitrogen, Carlsbad, CA) consisting of 10μg/L human recombinant EGF (Invitrogen), 100μg/L cholera toxin (Calbiochem; Merck KGaA, Darmstadt, Germany), antibiotics, and growth supplement supplied by the manufacturer was supplemented. Medium was changed every 3 to 4 days. Within 10 days, the explant was carefully transferred to a new dish and cultured as described above. Subculture was performed using explants culture method similar to Ma *et al* The mouse corneal epithelial cells (MCEs) were subcultured by TrypLE Express (Invitrogen) at 1:3 split after small cells reached subconfluence until passage 4 (P4) cultures. From P5, MCEs after subconfluence were subsequently serially passage at a density of 5×10^4 per 75-cm^2 flask, 7-10 days per passage. The cultures were incubated respectively in normoxia (210mL/L O_2 and 50 mL/L CO_2) or in hypoxia (20mL/L O_2 and 50 mL/L CO_2) with the medium changed every 3 to 4 days.

Methods

Colony forming efficiency MCEs were inoculated in 60mm dishes at 1000 cells/dish and cultured for 10 days. Cultured cells were stained with eosin for 1 hour. Colony forming efficiency (CFE) was calculated as the number of colonies/number of inoculated cells. Images were scanned,

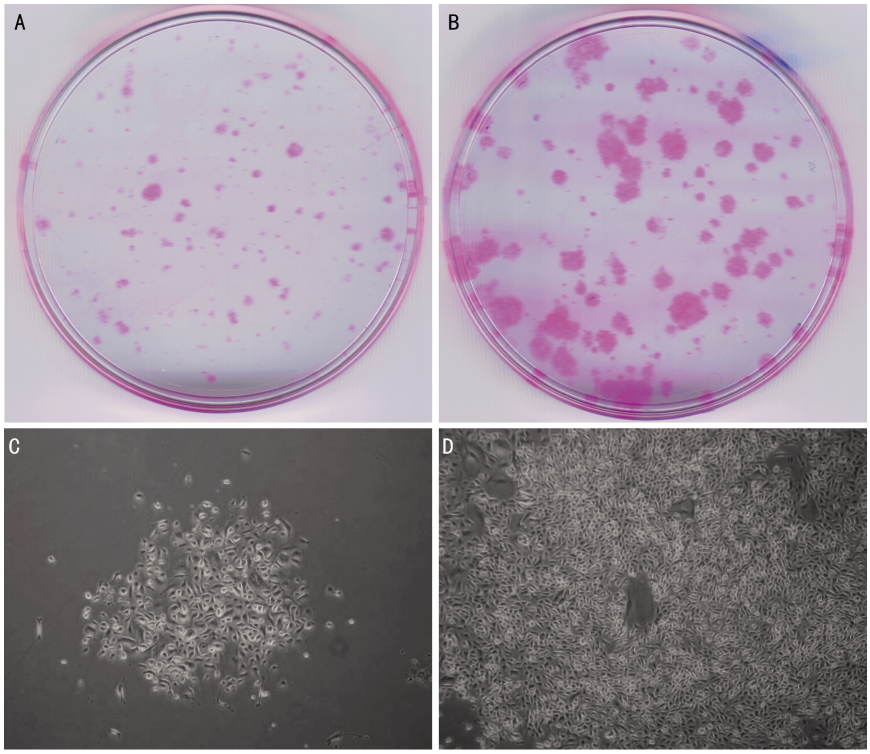

Figure 1 CEF cultures A: Normoxia; B: Hypoxia; C: Normoxia; D: Hypoxia

and eosin-stained area was measured using Scion Image software (Scion Corp., Frederick, MD). Five independent experiments were performed.

Cell proliferation MCE proliferation was observed in 24-well plates initially seeded with 1000 cells per well. Two wells in each condition were fixed with 70% ethanol every 2 days for up to 20 days. Medium was changed every 2 days. After all wells were fixed, the plates were stained with eosin for 1 hour. Images were scanned, and eosin-stained area was measured using Scion Image software. Three independent experiments were performed.

Immunostaining MCEs (1×10^3cells/well) were cultured in gelatin-coated, 2-well chamber slides, respectively in normoxia or in hypoxia, and fixed with 40g/L para-formaldehyde (PFA, Wako Ltd. Osaka, Japan). PFA- fixed cells were permeabilized with 3g/L Triton X-100 (Sigma-Aldrich, St. Louis, MO). After blocking with 100mL/L normal donkey serum, the cells were treated with the following monoclonal primary antibodies: anti-p63 (1:100, 4A4; Santa Cruz biotechlogy, Inc., Santa Cruz, CA) and anti-K19 (1:100, NeoMarkers for Lab Vision Corporation, Fremont, CA). The cells were then treated with Cy3-conjugated secondary antibodies (Chemicon International, Inc., Temecula, CA). The nuclei were counterstained with 4', 6'-diamino-2-phenylindole (1mg/L, DAPI; Dojindo Laboratories, Tokyo, Japan).

Statistical Analysis Student's t-test was used to evaluate the difference between hypoxia and normoxia. $P<0.05$ was considered statistically significant.

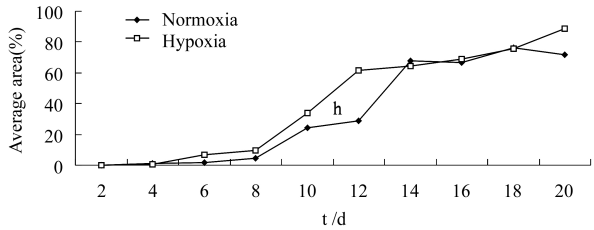

Figure 2 Cell growth curve in cultures

RESULTS

After 12 days, the normoxic colonies (Figure 1A, C) were smaller compared with colonies formed in hypoxia (Figure 1B, D). When the area covered by epithelial cells was used as a measure of cell proliferation, we found that the average area covered by the cells was (3.12±0.17)cm^2 in hypoxia and (0.84 ±0.04)cm^2 in normoxia and there was significant difference between them (n=5, $P<0.05$). In addition, colony-forming efficiency was (12.50±1.50)% in hypoxic cultures, which was similar compared with normoxia cultures [(11.13±1.86),n =5, $P>0.05$].The hypoxic cultures reached confluence faster than normoxic cultures. The average area covered by the cells in hypoxia was significantly higher after 12 days until the cells became confluent (Figure 2). The MCE expressed progenitor markers p63 and K19 in most of the cells under both normoxia and hypoxia (Figure 3).

DISCUSSION

As an essential micronutrient for cells, oxygen has a central role in oxidative phosphorylation, is a required cofactor for many enzymatic reactions, and modulates signal transduction pathways. The responsiveness of cells to oxygen tension in

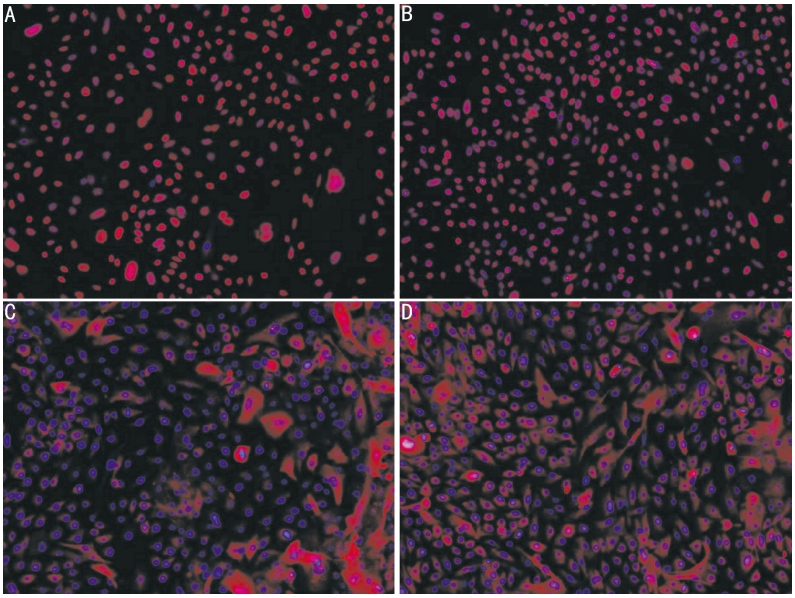

Figure 3 Immunostaining (×400) A: p63 expression in normoxia; B: p63 expression in hypoxia; C: K19 expression in normoxia; D: K19 expression in hypoxia

tissue culture is a physiological factor now receiving considerable attention. Except for the airways, the partial pressure of oxygen in tissues internally is often estimated as 5%, near that of the venous blood supply. This level is considerably below the 21% found in ambient air. The ambient oxygen concentration is not the normal oxygen concentration experienced by most cells in the human body. Finding an appropriate oxygen level is important to model proper behavior in vivo. Recently, the use of hypoxic incubation was reported to enhance progenitor cells in the bone marrow, neural cells, and hES cells.

However, unlike many internal tissues, the cornea has direct access to atmospheric oxygen. Cell culture at ambient oxygen conditions appears physiologically relevant to corneal epithelial cells. Hence, in the study, we investigated whether hypoxia can also be used to induce immature cells to expand from the murine limbus and enhance the expansion of epithelial progenitor cells *in vitro*. We found culturing mouse corneal epithelial cells in 2% O_2 produced larger colonies than did culturing in normoxia. We further observed the effects of hypoxia on cell proliferation, and found that cell growth was enhanced in hypoxia giving rise to larger initial colonies that reached confluence earlier than normoxic cultures.

Furthermore, we detected progenitor cell markers, nuclear transcription factor p63 and K19. The corneal epithelial progenitor cells including limbal stem cells (LSC) and transient amplifying cells (TAC). The expression of p63 and K19 [12] was localized to progenitor cells with high proliferative capacity, including both LSC and TAC at present. The results of immunostaining showed cells in both normoxia and hypoxia expressed p63 and K19 strongly, which indicates that the phenotype of the cells is equivalent to corneal epithelial progenitor cells. We found that limbal epithelial progenitor cells can be efficiently expanded in hypoxic conditions. This protocol may have an impact on the way ex vivo expansion is performed in the future. For example, the high yield of cells with high proliferative potential may allow the engineering of transplantable epithelial sheets from even a single cell source. However, the mechanisms that hypoxia enhance the expansion of epithelial progenitor cells are still not clear and further studies are needed to resolve this question.

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