·Basic Research·

Effect of calcium on the proliferation and differentiation of murine corneal epithelial cells *in vitro*

Xiao-Li Ma, Han-Qiang Liu

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Department of Ophthalmology, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

Correspondence to: Xiao-Li Ma. Department of Ophthalmology, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China. xiaolimax@hotmail.com

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Abstract

• AIM: To investigate the effect of calcium on the proliferation and differentiation of murine corneal epithelial cells *in vitro*.

• METHODS: Mouse corneal epithelial cells were cultured in serum-free low-Ca^{2+} medium (KSFM) and KSFM supplemented with 0.9mmol/L Ca^{2+}. Population doublings (PDs) were determined. The expression of corneal epithelial cell markers p63, keratin 19 (K19) and involucrin was investigated by RT-PCR analysis and semiquantitative analysis of Western blotting.

• RESULTS: Cells in KSFM were stably subcultured over 25 passages, however, none of the cell lines could pass P4 in KSFM with Ca^{2+}. In KSFM, the cells was were homogeneous and small cells with typical cobblestone appearance; and expressed p63, K19 and involucrin. After medium was supplemented with calcium, cells became a heterogeneous mix of small and large cells. Furthermore, semiquantitative analysis of Western blotting showed that the expression of involucrin was increased significantly.

• CONCLUSION: Calcium has the effect of inhibiting proliferation and triggering differentiation on mouse corneal epithelial cells.

• KEYWORDS: calcium; cornea; epithelium; cell culture

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INTRODUCTION

The corneal epithelium is an avascular transparent stratified squamous epithelium covering the external surface of the cornea. Similar to other epithelia, it needs to be constantly renewed. Corneal epithelial stem cells in limbus divide to produce daughter transient amplifying (TA) cells that proliferate, migrate, and differentiate to replace corneal epithelial cells physiologically shed during normal homeostasis or during recovery from an injury to the corneal epithelium. 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 [1]. Maintaining progenitor cells with high proliferative potential is one of the keys of successful limbal epithelial cell culture. It is well known that calcium trigger differentiation in several lines of epithelial cells [2,3]. We therefore investigated the effect of calcium on the expansion and the differentiation of epithelial progenitor cells from the murine limbus *in vitro*.

MATERIALS AND METHODS

Materials C57BL/6 mice (CLER, Tokyo, Japan), aged 8-10 weeks old, 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 phosphate buffered saline (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* [4]. 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. After approximately 5-10 minutes to allow for attachment of the explant. The cultures were incubated at 37℃, under 950mL/L humidity and 50mL/L CO_2 with the medium changed every 3 to 4 days. Within 10 days, the explant was carefully transferred to a new dish and cultured as described above. The mouse corneal epithelial cells (MCE) were subcultured using method similar to Ma *et al* [5]. MCE was 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 /75-cm^2 flask, 7-10 days per passage. The medium was changed

Table 1 Primers used for RT-PCR

Primer	Sequence (5'→3')	Product Size (bp)
p63	GTCAGCCACCTGGACGTATT ACCTGTGGTGGCTCATAAGG	321
Keratin 19	TGATCGTCTCGCCTCCTACT GGCTCTCAATCTGCATCTCC	356
Involucrin	CAAGACATGCTAGTACCACAGG GTGTCCGGTTCTCCAATTCGTG	883
β-actin	TGTTACCAACTGGGACGACA TCTCAGCTGTGGTGGTGAAG	392

every 3 to 4 days. In order to investigate the effect of calcium on differentiation the cells, we cultured the cells in 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, and KSFM supplemented with 0.9mmol/L Ca^{2+} (KSFM+Ca) in primary culture and subculture, respectively. The primers used for RT-PCR in this study are shown in Table 1.

Methods The population doublings (PDs) were calculated as $\log_2 (D/D_0)$, where D and D_0 were defined as the density of cells at the time of harvesting and seeding, respectively. Total RNA was extracted from MCEs cultured for 7 to 10 days using commercial RNA isolation kit (RNeasy, Qiagen, Valencia, CA), and cDNA was synthesized using kit (RevaTra Ace, Toyobo, Osaka, Japan). The same amount of cDNA was amplified by PCR (GeneAmp 9700; Applied Bioscience, Inc., (ABI), Foster City, CA) for each primer pairs (Table 1). PCR products were analyzed by agarose gel electrophoresis. Other MCE was dissolved with lysis buffer (M-PER, Pierce, Rockford, IL). Same amount of proteins were loaded on a 10% Bis-Tris gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were immunostained with primary antibodies against p63 (1:200), K19 (1:50), involucrin (1:3000) and β-actin (1:500, mabcam 8226; Abcam Inc., Cambridge, MA), respectively. After the reaction with horseradish-peroxidase conjugated secondary antibody, protein bands were visualized by ECL (GE Healthcare, Buckinghamshire, UK) and X-ray film.

RESULTS

Population Doubling of cells The cells cultured in KSFM and KSFM with high Ca^{2+} were cultured from explant (P0), subcultured though 5 passages in KSFM for 11.53±1.74 PDs (mean ±SD, n=6, Figure 1) and were stably subcultured though at least 25 passages without showing signs of replicative senescence (not shown). However, none of the cell lines could pass P4 in KSFM with high Ca^{2+} (n=6, Figure 1).

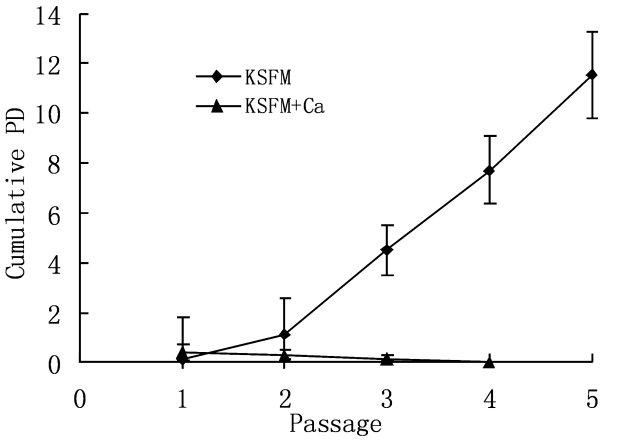

Figure 1 Population doubling (PD) of cells cultured in KSFM and KSFM+Ca

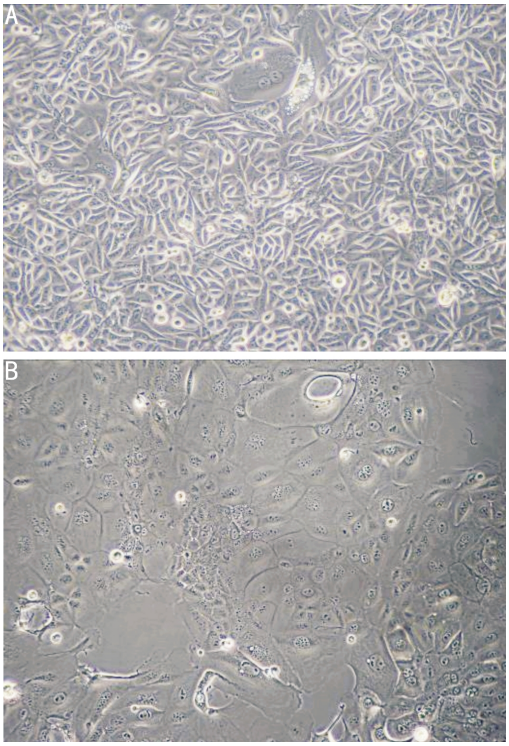

Figure 2 Morphology of cells cultured in A: KSFM; B: KSFM with high Ca^{2+}

Cell Morphology The cells cultured in KSFM were homogeneous small cells with a uniform cobblestone apperance (Figure 2A), whereas cells in KSFM with high Ca^{2+} were a heterogeneous mix of small and large cells (Figure 2B).

Cell Marker Cells expressed progenitor markers P63 and K19 and differentiation marker involucrin. After medium was supplemented with high Ca^{2+}, the expression of differentiation marker involucrin was increased significantly (Figure 3A,B). Moreover, semiquantitative analysis of Western blots showed that the ratio of involucrin to β-actin was 5.92±3.70% in KSFM compared to 33.05±9.02% in KSFM with high Ca^{2+}, and there was significantly difference between them (P<0.005, n=6, Student's t-test,).

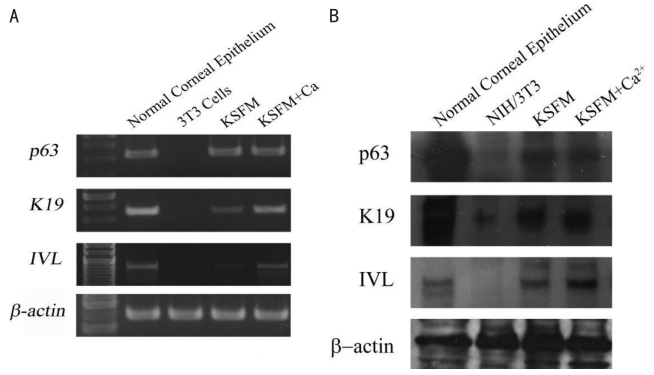

Figure 3 p63, K19 and IVL expression in different medium
A: RT-PCR; B: Western blotting

DISCUSSION

Damage to limbal stem cells through inflammation, infection, injury, drug toxicity, and postoperational complications can cause loss of vision as a result of inflammation and scarring, vascularization, and conjunctival cell ingrowth. Corneal transplantation for patients with severe ocular surface disease performed along with limbal epithelial grafting has shown significant improvement in the outcome of the surgery[6]. The conventional limbal epithelial transplantation requires harvesting many stem cells from a donor, such as cadaveric, live-related donor and the host himself, which puts the donor eye at high risk of losing the viability of its own corneal epithelium or/and requires the host indefinite immunosuppression to avoid limbal allograft rejection. Tissue engineering techniques involving ex vivo expansion of limbal epithelial tissue starting from a population of cells harvested from a small limbal biopsy have overcome this hurdle and have shown tremendous potential [7]. A central quest in the field of ocular surface bioengineering is to optimize culture condition to maintain progenitor cells with high proliferative potential. In our previous study, we found mouse corneal epithelial cells were stably subcultured though at least 25 passages without showing signs of replicative senescence in KSFM, however, none of the cell lines could pass P4 in SHEM (supplementary hormonal epithelial medium，not shown). Furthmore, we analyzed the difference between KSFM and SHEM. We found the concentration of calcium was different in the two medium and the concentration of calcium in SHEM was significantly higher than KSFM [5]. It is well known that calcium trigger differentiation in several lines of epithelial cells [2,3]. Therefore, we investigated the influence of calcium on the expansion and the differentiation of epithelial progenitor cells from the murine limbus *in vitro*. We found that the cells were stably subcultured though at least 25 passages without showing signs of replicative senescence in KSFM but none of the cell lines could pass P4 in KSFM with high Ca^{2+}. In morphology, the cells cultured in KSFM were homogeneous small cells with a uniform cobblestone appearance, whereas cells in KSFM with high Ca^{2+} were a heterogeneous population of small and large cells, which indicated calcium could inhibit proliferation and trigger differentiation of murine epithelial cells.

Furthermore, we detected progenitor cell markers, including nuclear transcription factor p63 and K19, and differentiation marker involucrin[8]. The expression of p63[9] and K19[10] was localized to progenitor cells with high proliferative capacity, including both LSCs and TACs at present. The results of RT-PCR and Western blotting showed cells in low-Ca^{2+} medium KSFM expressed p63 and K19 strongly, which indicates that the phenotype of the cells might be equivalent to corneal epithelial progenitor cells. After medium was supplemented with calcium, the expression of differentiation marker involucrin was increased significantly, which confirmed Ca^{2+} can induce the murine corneal epithelial cells to differentiate.

In conclusion, we compared the proliferation and differentiation of mouse corneal epithelial cells in low-Ca^{2+} medium and medium supplemented with calcium (0.9mmol/L Ca^{2+}). Calcium has the effect of inhibiting proliferation and triggering differentiation on mouse corneal epithelial cells. Low-Ca^{2+} medium is a preferable option to culture mouse corneal epithelial cells. However, the mechanisms that calcium trigger differentiation of murine epithelial progenitor cells are still not clear and further studies are needed to resolve this question.

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