·Basic Research·

Effect of fetal bovine serum on the proliferation and differentiation of murine corneal epithelial cells *in vitro*

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Foundation item: Liaoning Provincial Department of Education, China (No.05L564)

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Received:2009-08-22 Accepted:2009-10-12

Abstract

• AIM: To investigate the effect of fetal bovine serum (FBS) on the proliferation and differentiation of murine corneal epithelial cells *in vitro*.

• METHODS: Mouse corneal epithelial cells (MCEs) were cultured in serum-free low-Ca^{2+} medium (KSFM) and KSFM supplemented with 100mL/L FBS, respectively. Population doublings (PDs) were determined. The expressions of corneal epithelial cell markers p63, keratin 19 (K19) and involucrin were investigated by RT-PCR and Western blotting analyses.

• RESULTS: Cells in KSFM were stably subcultured over 25 passages; however, none of the cell lines could pass P3 in KSFM with FBS. In KSFM, the cells showed typical cobblestone appearance and expressed p63, K19 and involucrin. After medium was supplemented with FBS, cells became homogeneous, large and squamous. Furthermore, both RT-PCR and Western blotting analyses showed that the expression of involucrin was increased significantly.

• CONCLUSION: FBS has effects of inhibiting proliferation and triggering differentiation of MCEs.

• KEYWORDS: serum; cornea; epithelium; cell culture

Ma XL, Que YH, Kong J, Liu HQ, Zhang JS. Effect of fetal bovine serum on the proliferation and differentiation of murine corneal epithelial cells *in vitro*. *Int J Ophthalmol* 2009;2(4):298-301

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 [1]. Maintaining progenitor cells with high proliferative potential is one of the keys of successful limbal epithelial cell culture. It is well known that serum triggers differentiation in several lines of epithelial cells[2,3]. We, therefore, hypothesized that serum-free medium could be used to enhance the expansion of epithelial progenitor cells from the murine limbus *in vitro*.

MATERIALS AND METHODS

Tissue Preparation and Cell Culture 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. After sacrificing the mice, eye globes were enucleated with forceps and 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 extraneous tissue was cleaned away (e.g., iris and ciliary body). Primary cell culture was performed using an explant culture method similar to Hazlett *et al* [4,5]. Briefly, the button was cut in half, and each explant with epithelium side up was plated flat on a 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 95% 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.

Subculture The mouse corneal epithelial cells (MCEs)

were subcultured by TrypLE Express (Invitrogen) at 1:3 split ratio after small cells reached subconfluence until Passage 4 (P4) cultures. From P5, MCEs after subconfluence were subsequently serially passed at a density of 5×10^4 per $75cm^2$ flask, 7-10 days per passage. The medium was changed every 3 to 4 days.

Medium In order to investigate the effect of serum on the differentiation of cells, we cultured the cells in serum-free low-Ca^{2+} medium (designated "keratinocyte serum-free medium", KSFM; Invitrogen, Carlsbad, CA) consisting of 10ng/mL human recombinant epidermal growth factor (EGF) (Invitrogen), 100ng/mL cholera toxin (Calbiochem; Merck KGaA, Darmstadt, Germany), antibiotics, and growth supplement supplied by the manufacturer, and KSFM supplemented with 100mL/L fetal bovine serum (FBS) (KSFM+FBS) in primary culture and subculture, respectively.

Cell Proliferation 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.

Reverse Transcription –Polymerase Chain Reaction 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 the RevaTra Ace kit (Toyobo, Osaka, Japan). The same amount of cDNA was amplified by PCR (GeneAmp 9700; Applied Bioscience, Inc. (ABI), Foster City, CA) for each primer pair, as shown in Table 1. PCR products were analyzed by agarose gel electrophoresis.

Western Blotting Analysis MCEs were dissolved with lysis buffer (M-PER, Pierce, Rockford, IL). The same amount of proteins were loaded on a 100mL/L 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 (1:3 000) 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 (PDs) of cells cultured in KSFM and KSFM+FBS was shown in Figure 1. The cells were cultured from explant (P0), subcultured though 5 passages in KSFM for 11.53±1.74PDs (mean±SD, n=6, Figure 1) and were stably subcultured though at least 25 passages without showing signs of replicative senescence (data not shown). However, none of the cell lines could pass P3 in KSFM+FBS (n=6, Figure 1).

Table 1 Primers used for RT-PCR

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

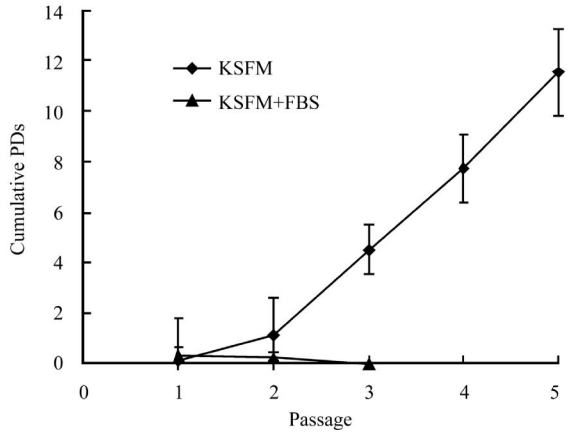

Figure 1 Population doublings (PDs) of cells cultured in KSFM and KSFM+FBS The cells were subcultured though 5 passages in KSFM (n=6) and were stably subcultured though at least 25 passages (data not shown). However, the cells could not pass P3 in KSFM+FBS (n=6)

With respect to morphology, the cells cultured in KSFM were homogeneous small cells (Figure 2A), while cells in KSFM+FBS were homogeneous, large and squamous (Figure 2B), indicating that FBS quickly increases the size of the cells.

In KSFM, both RT-PCR and Western blotting analysis showed that cells expressed progenitor markers p63 and K19 and differentiation marker involucrin (IVL). After medium was supplemented with FBS, the expression of differentiation marker involucrin was increased significantly (Figure 3A, 3B).

DISCUSSION

FBS is frequently added to the defined basal medium as a source of certain nutritional and macromolecular growth factors essential for cell growth. However, it is well known that FBS triggers differentiation in several lines of epithelial

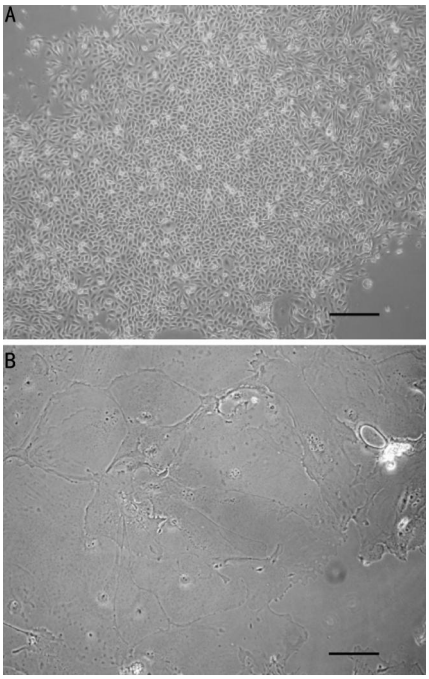

Figure 2 Induction of differentiation by switching KSFM (A) to KSFM+FBS (B). Scale bars: 100μm

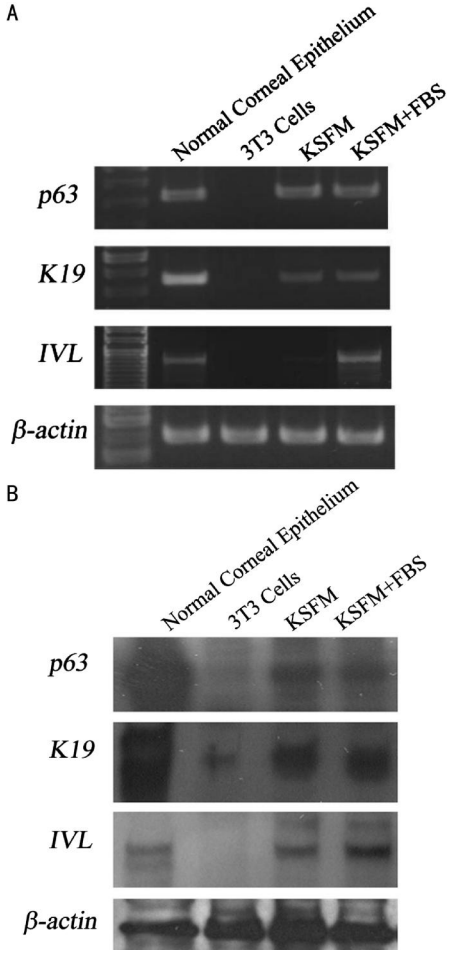

Figure 3 RT-PCR (A) and Western blotting (B) analyses of p63, K19 and IVL expressions in different media. Normal mouse epithelial cells were used as a positive control, and fibroblast cell line NIH/3T3 cells were negative control

cells [2,3]. Therefore, in order to investigate the effect of FBS on proliferation and differentiation of corneal epithelial cells and optimize the culture method, we compared the culture of MCEs in serum-free medium (KSFM) and medium with FBS (KSFM+FBS).

The corneal epithelium is a rapidly regenerating stratified squamous epithelium. The epithelial progenitor cells, including limbal stem cells (LSCs) and transient amplifying cells (TACs) [6] with extensive proliferative potential, are crucial for maintaining the homeostasis of corneal epithelium, which is an important prerequisite not only for the integrity of the ocular surface but also for visual function. Romano *et al*[7] have reported that the limbal basal epithelium has the smallest cell size in the corneal epithelial differentiation scheme. In our study, the cells cultured in KSFM appeared to have similar morphology, characterized by homogeneous small cell size with typical cobblestone appearance, which suggests that the cells maintain their progenitor cell state. However, after medium was supplemented with FBS, the size of the cells increased quickly and became homogeneous, large and squamous (Figure 2B), which suggested that FBS induces the differentiation of 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 the presence of both LSCs and TACs. The results of RT-PCR and Western blotting showed that cells in KSFM expressed p63 and K19 strongly, which indicates that the phenotype of the cells might be equivalent to corneal epithelial progenitor cells. However, after medium was supplemented with FBS, the expression of differentiation marker involucrin was increased significantly, which confirmed that FBS triggers the differentiation of corneal epithelial cells.

In conclusion, we compared the proliferation and differentiation of mouse corneal epithelial cells in serum-free medium and medium supplemented with FBS. FBS has the effect of inhibiting proliferation and triggering differentiation of mouse corneal epithelial cells. Therefore, serum-free medium is a preferable option to culture mouse corneal epithelial cells.

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