·Basic Research·

Effect of basic fibroblast growth factor on cat corneal endothelial cell proliferation

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Received:2011-03-04 Accepted:2011-06-02

Abstract

• AIM: To investigate the function of basic fibroblast growth factor (bFGF) on cat corneal endothelial cells proliferation.

• METHODS: Cat corneal endothelial cells were primarily cultured, stimulated with bFGF for different period, the proliferation of cells was assayed by modified tertrozalium salt (MTT) method, and the morphologic changes were observed with inverted phase contrast microscope and transmission electron microscope.

• RESULTS: At 1, 3 and 5 days after bFGF was added to cat corneal endothelial cells, the result of MTT in 490nm showed significant difference than that in control group, and the difference was most significant in 10ng/mL group.

• CONCLUSION: bFGF can promote proliferation of cat corneal endothelial cells. 10ng/mL is the relatively most effective dose.

• KEYWORDS: basic fibroblast growth factor; corneal endothelial cells; modified tertrozalium salt assay

DOI:10.3980/j.issn.2222-3959.2011.04.12

Luo WJ, Zhou Y, Liu MG, Wang CF. Effect of basic fibroblast growth factor on cat corneal endothelial cell proliferation. *Int J Ophthalmol* 2011;4(4):384-387

INTRODUCTION

Corneal endothelial cells play an important role in keeping the transparency of the cornea, and the normal thickness and transparency of cornea can be kept through its regulation in the ion's density and moisture of cornea [1]. Once the corneal endothelial cells were damaged, the pumping function of the cells will be in disorder, which can lead to corneal edema. The rodent's and the rabbits' corneal endothelial cells have high reproducibility, the cats' and primates' have lower reproducibility, while the humans' have barely the function of division. A lot of scholars have tried to improve the proliferation of corneal endothelial cells by stimulating cell growth factors. In this experiment, the ultrastructure was detected by MTT, inverted microscope and transmission electron microscope (TEM) to observe whether bFGF accelerated the proliferation of cats' corneal endothelial cells cultured *in vitro* or not.

MATERIALS AND METHODS

Materials The main reagents included DMEM culture fluid and fetal bovine serum (GIBCO Co., USA) , bFGF (Promega, USA), and MTT (Fluka Co.). The main apparatus and devices included CO_2 incubator (MCO175, SANYO, Japan), an inverted microscope (Olympus, Japan) and an ELIASA (Sigma, USA).

Methods

Primary culture of cat´s corneal endothelial cells Sixty cats of 2 months old, no limit to sex, healthy without medical history, 120 eye balls, found no abnormality with examination. The cat's eyeballs were removed, washed by normal saline and soaked in antibiotics D-Hanks fluid which included penicillin 100U/mL and streptomycin 100μg/mL for 30 minutes. The cornea was extracted along the limbal on the super clean bench, descemet membrane with corneal endothelial cells was torn under the operating microscope, and put in a petri dish containing 0.25% pancreatic protein enzyme for digestion, then incubated at 37℃ for about 15 minutes. DMEM with 10% fetal bovine serum was added into the petri dish to terminate the digestion when the endothelial cells were loose and before falling, the cells were collected after being centrifuged at 1 000r/min for 10 minutes, then inoculated in a culture flask and cultured in an incubator with 5% CO_2 at 37℃. About 2 weeks later, when the cells fused into single layer, they were digested by 0.25% trypsin, counted and made into 5.0×10^4/mL cell suspension. Then it was inoculated in the incubator with 5%

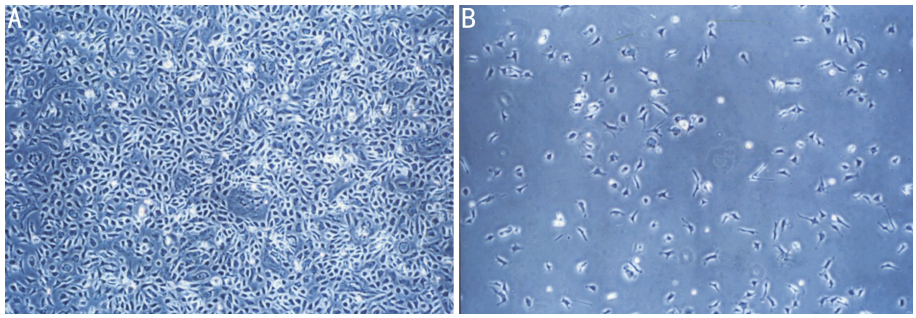

Figure 1 Cells cultured for 120 hours (×100) A: bFGF 0.01g/L group; B: Control group

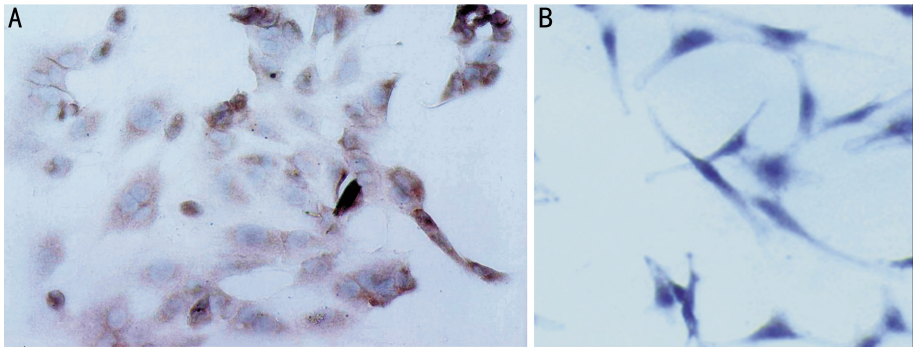

Figure 2 A: NSE staining was positive in cat endothelial cells(×400); B: Corneal stromal cells had no specific antigen staining (×400)

CO_2 at 37℃ . The cells adhered after approximately 24 hours. The original culture medium was removed, conditioned medium were added in, and the cells were incubated for 1-5 days sequentially.

Identification of cat´s corneal endothelial cells by immunohistochemical staining The digested cells with the density of 1×10^4 per well were inoculated in the 6-well petri dish paved with glass slides. Immunohistochemical staining of NSE was performed after 24 hours. Brown-yellow particles were found in the cytoplasm of the corneal endothelial cells. Corneal stromal cells were used as the comparison.

Experimental groups DMEM culture medium containing 0.5% FCS was set as control group, bFGF diluted by DMEN with 0.5% FCS as experimental group. The density of each group was 1×10^{-3}g/L, 0.01g/L and 0.1g/L. Periods of observation were set as 1, 3, 5 days, and 10 wells were observed in each group.

Determination by MTT Conditioned medium was added in each group, incubation was performed in accordance with different periods of each group, MTT solution (5g/L) 20μL was added in each group, which was constantly incubated in the incubator for 4 hours with 5% CO_2 at 37℃ . Incubation was terminated, culture medium was sucked out and removed from the wells. Each well was added with 150μL DMSO and oscillated by using micromixer for 10 minutes. OD value of each well was tested in turn by ELISA, and the wevelength was 490nm.

Morphology observation The growth of corneal endothelial cells were observed with the help of inverted phase contrast microscope regularly. Cells growing for 5 days in the conditioned medium of each group were taken microphotographs. Detection to the ultrastructure was performed using the TEM after 2.5% glutaraldehyde was fixed.

Statistical Analysis The MTT results were analyzed using software SPSS 11.0. The mean ±SD was calculated and Student's t test was performed in accordance with different density and time points of the results.

RESULTS

Observation of cats´ corneal endothelial cells by inverted phase contrast microscope Cells adhered after 24 hours' primary culture, and expanded to be massive single layer cells which were types of similar circular and polygon. Passage cells were inoculated in 96-well board for 24 hours, and most of them adhered. Each experimental group was added with bFGE and cultured. Cells proliferated sooner compared to control group at the first 24 hours; after 72 hours, cells proliferated as shapes of roundness and polygon with small scales, but less in control group. After 120 hours, cells in experimental group proliferated in massive as the shape of regular triangular to hexagonal, cell boards were clear (Figure 1A), while significantly fewer in control group (Figure 1B).

NSE Staining Brown-yellow thick particles appeared in the cytoplasm around the nucleus of the cat's corneal endothelial cells. The positive rate was over 98% (Figure 2A). Corneal stromal cells had no specific antigen staining (Figure 2B).

Effects of bFGF on the proliferation of cat´s corneal endothelial cells detected by MTT The MTT increment was elevated in each experimental group 24-120 hours with the effects of bGFG density, and the increment would increase as the growth of time. The difference of the MTT increment was significant (P<0.01). Among the three density values, 0.01g/L was the most effective one, while the 0.1g/L was depressed(Figure 3,Table 1).

Detection of ultrastructure by TEM Added with culture medium 120 hours later, cells of the bFGF groups were observed by the TEM. Cell shape was plump, abundant organelles in the cytoplasm were observed, the cells grew well (Figure 4).

DISCUSSION

High transparency and optics are the essential conditions to keep the humans' visual organ functioning appropriately. The function of physical barriers and pumping for the metabolism of healthy and enough qualified corneal endothelial cells are the preconditions for keeping transparency, the normal thickness and semi-dehydration of the cornea. Long-term researches have indicated that the corneal endothelial cells of adult cats and primates, especially the humans have no the ability to proliferate any more. When they are damaged, the coloboma is filled through the expansion and migration of the adjacent cells, while the compensatory capacity was limited. When the cell density decrease to 25%-45% of the normal value, hexagon cells are less than 30% -40% , the single cell's acreage increased to 3-4 times caused by the damage of corneal endothelial cells, plenty of clinical keratopathy happens, such as decrease of the transparency, inflammatory response of anterior chamber or edema of the corneal stromal caused by mechanical scrape wound during intraocular operations, and bullous keratopathy can be caused in severe cases[3,4]. Therefore, to explore the pathway for improving the proliferation of corneal endothelial cells has been a hot spot in ophthalmology. Experimental results in recent years have indicated that humans' and primates' corneal endothelial cells may have stronger capacity of proliferation and physiologic compensation, compared to the traditional concept[5,6]. However, in the condition of normal physiology, humans' and primates' corneal endothelial cells have no capacity to proliferate, this is related to cytokines of the cells formed by themselves. Once the living environment of corneal endothelial cells deviate away from the normal condition, the possibility of proliferation may increase. Therefore, many scholars have tried to improve the proliferation of corneal endothelial cells using the cytokines.

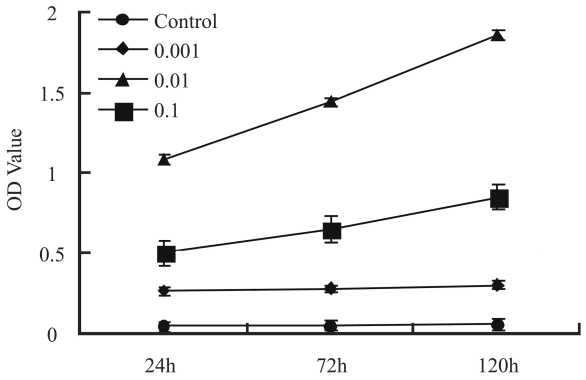

Figure 3 Effects of bFGF in various concentration cultured CECs by MTT chromatometry

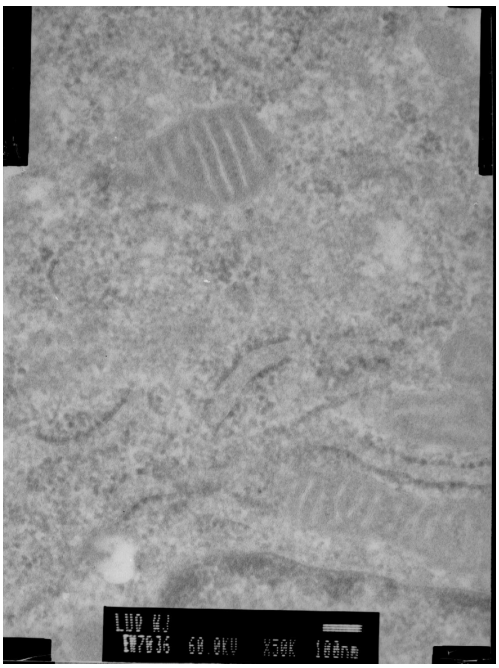

Figure 4 Cells cultured for 120 hours from bFGF 10ng/mL group in transmission electron microscope(×50K)

Table 1 Effects of bFGF in various concentration cultured CECs by MTT chromatometry (g/L, mean±SD)

Hours	Control	bFGF		
		1×10^{-3}	0.01	0.1
24	0.040±0.028	0.263±0.013	1.086±0.017	0.501±0.045
72	0.049±0.031	0.277±0.012	1.437±0.031	0.644±0.079
120	0.053±0.020	0.301±0.024	1.853±0.012	0.853±0.053

Cell factors, which are generated by the active cells in the hematopoietic system, the immune system or the inflammatory reaction, have the abilities to regular the proliferation and differentiation and induce cells to make their function. The cell factors are high-activity and multifunctional peptide, protein or glycoprotein, and improve the growth and development of cells through the binding to the receptors of the specific cell factors in the

target cell membrane. The EGF, FGF and PDGF closely relate to the cornea, and are studied much.

Of the mammal, FGF belongs to multi-functional growth regulating family. Unlike other growth factor families, one of the distinguishing features of FGF is the high affinity to heparin. FGF exists in the humans' and animals' eyes, and bFGF has primarily effects on stimulating the mitosis, chemotaxis, migration and repair of injury and can also change the shape of corneal endothelial cells, improve the differentiation and chemotaxis of corneal endothelial cells, stimulate the repair of damage of the living and isolated corneal endothelial cells.

In the experimental, bFGF's effects on corneal endothelial cells were confirmed by MTT method. Among the three set density, 1×10^{-3}g/L, 0.01g/L and 0.1g/L, the priliferative reached a maximum when the density was 0.01g/L. When the dosage increase to 0.1g/L, on the contrast the proliferative decreased. This was caused by the limited qualities of the bFGF receptors on cells surface. When the density of 0.01g/L was added, bFGF receptors had already combined enough bFGF and reached saturated condition. When the density increased further, the increased bFGF could restrain the combination with the receptor and could not improve the proliferative effects, while the uncombined and surplus bFGF would be stored in ECM. Under the inverted phase microscope and TEM, observation of the cells in control group and bFGF groups was performed, the promotion effect of proliferation of corneal endothelial cells by bFGF was also proved in morphology.

The experimental results proved the theory basis for the study of culture *in vitro* and repair of corneal endothelial cells.

REFERENCES

1 Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS. Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. *Transplantation* 2011;91(8):811-819

2 Luo WJ, Zhao GQ,Wang CF,Wang XJ. Cloning, expression and functional analysis of human PDGF-B chain peptide on cat corneal endothelial cell trauma reconstruction. *Chinese Trauma Research* 2009;12(1):31-37

3 Rose L, Kelliher C, Jun AS. Endothelial keratoplasty: historical perspectives, current techniques, future directions. *Can J Ophthalmol* 2009;44(4):401-405

4 Ghosheh FR, Cremona FA, Rapuano CJ, Cohen EJ, Ayres BD, Hammersmith KM, Raber IM, Laibson PR. Trends in penetrating keratoplasty in the United States 1980-2005. *Int Ophthalmol* 2008;28(3):147-153

5 Schroeter J, Rieck P. Endothelial evaluation in the cornea bank. *Dev Ophthalmol* 2009;43:47-62

6 Pels E, Rijneveld WJ. Organ culture preservation for corneal tissue. Technical and quality aspects. *Dev Ophthalmol* 2009;43:31-46

7 Xia L, Zhao M, Xu M. Experimental investigation of pEGFP-bFGF gene transfer to human limbal stem cells. *Shengwu Yixue Gongchengxue Zazhi* 2009;26(1):148-152