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Mechanism of induction of fibroblast to corneal endothelial cell

Yan Jiang, Wei-Cai Fu, Lin Zhang*

Department of Ophthalmology, Renji Hospital Affiliated to Medical College of Shanghai Jiaotong University, Shanghai 200127, China

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ABSTRACT

Objective: To explore mechanism of nduction of fibroblast to corneal endothelial cell. **Methods:** Rabbit conjunctiva fibroblasts were used as feeder cells, rabbit oral mucosa epithelial cells were used as seed cells, and human denuded amniotic membrane was used as carrier to establish tissue engineering corneal endothelium. The transformation effect was observed. **Results:** As concentration of mitomycin C increased, cell survival rate gradually decreased, cell proliferation was obviously inhibited when concentration≥25 μg/mL; 5 days after being treated by 5 μg/mL mitomycin C, cell body was enlarged and extended without cell fusion, however after being treated by 0.5 μg/mL mitomycin C, cell body was significantly proliferated and gradually fused; after 3 weeks of culture, stratified epithelium appeared on rabbit oral mucosa epithelial cells, differentiation layers were 4-5 and were well differentiated, the morphology was similar to corneal endothelial cells; Under electron microscope, surface layer of cells were polygonal, tightly connected to another with microvilli on the border, there was hemidesmosome between basal cells and human denuded amniotic membrane. **Conclusions:** Fibroblast cells have the potential of multi-directional differentiation, effective induction can promote emergence of intercellular desmosomes between seed cells and emergence of epithelial surface microvilli, and differentiate to the corneal endothelial cell. However, clinical application still needs more research and safety evaluation.

1. Introduction

At present, there are 1 million blind patients caused by injury of corneal epithelial cell function every year, the only treatment for the disease is corneal endothelium transplantation. However, the amount of donor cornea is very limited clinically[1]. Transplantation of contralateral healthy cornea will cause injury of the healthy corneal limbus tissue, which is irreversible[2,3]. Transplantation of transformed mouse embryonic tissue fibroblasts may bring risk of heterogenous pathogen spread caused by heterogenous source cells[4]. Thus, inducing autologous fibroblasts to transform to corneal endothelium cells is the main research direction for treatment of corneal endothelium disease.

*Corresponding author: Lin Zhang, Chief Physician, Professor, Doctoral Supervisor, Department of Ophthalmology, Renji Hospital Affiliated to Medical College of Shanghai Jiaotong University, Shanghai 200127, China.
E-mail: 28906390 @ qq.com
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2. Materials and methods

2.1. Reagents and equipments

DMEM/F12 culture medium and 60 mm culture dish were from Enzyme-linked Biological Technology Co., LTD., Shanghai; Fetal calf serum was from Weifang Ruihua Biotechnology Co., LTD; 6 well/96 well culture plate was from Shanghai Lingchu Environmental Protection Equipment Co., LTD; 0.25% pancreatin+ethylenediamine tetraacetic acid (EDTA), Human epidermal growth factor, insulin and dimethylsulfoxide (DMSO) were from Sigma Company, America; Embedded culture plate was from Corning Company, America; Mitomycin C was from Zhejiang Haizheng Pharmaceutical Co., LTD; Inverted microscope was from Olympus Optical Co Ltd; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco company, America; Super clean bench for cell culture was from Beijing Semiconductor Equipment Factory; Cell incubator was from Shanghai SANTN Instrument Co., LTD; Spectrophotometer was from PE Company, America; Centrifugal machine was from Shanghai Anting Scientific

Instrument Factory.

2.2. Establishment of feeder cells

2.2.1. Primary culture

Four healthy New Zealand white rabbit were taken (weighting around 2 kg and purchased from Lvmei warren), tissue explants[5-7] were used to take primary rabbit conjunctival fibroblast with size of 4 mm×4 mm. After being washed by normal saline for 3 times, conjunctival matrix organization was cut into 1 mm×1 mm pieces. Samples were placed in culture dish, fetal calf serum was added in after 10 min and cultured at room temperature and 5% CO_2 for 1 h. Then tissue was placed in DMEM/F12 culture medium, and continued culture at room temperature and 5% CO_2. Medium was changed every 48 h, conjunctiva matrix tissue sample was taken after 7 d. When the fusion rate of primary cells reached 90%, 0.25% pancreatin+EDTA were used for digestion, cells were subcultured with proportion of 1:3[8,9].

2.2.2. Treatment by mitomycin C

Mitomycin C was used to inhibit cell proliferation of 1-2 generation fibroblasts to explore the most appropriate treatment concentration. 0, 0.5, 5, 25, 50, 250, and 500 μg/mL concentrations of mitomycin C were used for incubation[10]. PBS buffer was used to rinse for three times after 2 h, then normal culture medium was added to culture at room temperature for 72 h. After culture, 150 μL DMSO was added in each well and shaken up. Spectrophotometer was used to detect optical density (OD) value. The third generation fibrolasts accepted the same treatment, besides, after being washed by PBS buffer for 3 times, 0.25% pancreatin+EDTA were used for digestion, centrifuged by 1 000 r/min for 5 min and then cell proliferation was observed[11]. We analyzed the more appropriated mitomycin C concentration, which was used to establish feeder cells.

2.3. Establishment of corneal endothelial cell

2.3.1. Establishing method

3 mm×3 mm of rabbit oral mucosa epithelial tissue was taken and washed to be placed under microscope. 1 mm×1 mm was cut after cleaning connective tissue and placed in embedded culture dish (Before that denuded amniotic membrane was placed in embedded culture dish). Culture medium in feeder cell culture plate was taken and placed in embedded culture dish, then 2 mL culture medium was added[12-15]. Ingredients of culture medium included DMEM/F12 culture medium at 1:1 or 3:1, 5%-10% fetal calf serum, 1-5 μg/mL insulin, 10-20 μg/mL human epithelial growth factor, 0.5% DMSO, and 1% antibiotics..

2.3.2. Morphological identification

Hematoxylin-eosin staining was applied to oral mucosa epithelial tissue after 3-week culture. The tissue was observed under microscope and treated referring to references[16,17], then observed under electron microscope.

3. Results

3.1. Result after mitomycin C treatment

As concentration of mitomycin C increased, cell survival rate gradually decreased, cell proliferation was obviously inhibited when concentration≥25 μg/mL (Table 1); 5 days after being treated by 5 μg/mL mitomycin C, cell body was enlarged and extended without cell fusion (Figure 1-A), however, after being treated by 0.5 μg/mL mitomycin C, cell body significantly proliferated and gradually fused (Figure 1-B). Thus 5 μg/mL was selected as the most appropriated mitomycin C concentration.

Table 1

Effects of different concentrations of mitomycin C on survival rate of rabbit conjunctival fibroblasts.

Concentration mitomycin C (μg/mL)	OD value	Survival rate(%)
0*	1.86±0.37	100.0
0.5	1.65±0.31	88.4
5	1.58±0.29	87.1
25	1.49±0.30	79.5
50	1.36±0.21	72.8
250	0.08±0.02	3.1
500	0.03±0.01	2.5

*Note: 0 μg/mL of mitomycin concentration was set as control of survival rate.

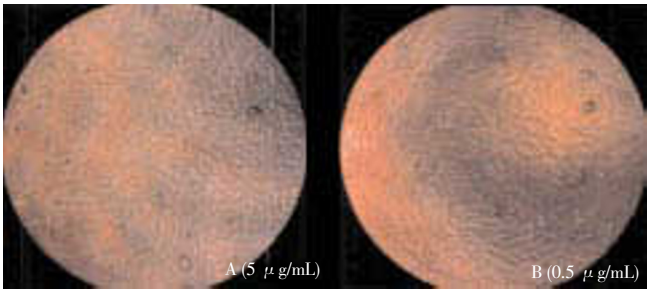

Figure 1. Effects of different concentrations of mitomycin C on proliferation and fusion of rabbit conjunctival fibroblasts(×100).

3.2. Histopathologic examination

After 3 weeks of culture, stratified epithelium appeared on rabbit oral mucosa epithelial cells, differentiation layers were 4-5 and were well differentiated, the morphology was similar to corneal endothelial cells (Figure 2).

Figure 2. Histopathologic image of rabbit oral mucosa epithelial cells after 3-week culture(×400).

3.3. Electron microscope examination

Under electron microscope, tightly connected to another

with microvilli on the border, there was hemidesmosome between basal cells and human denuded amniotic membrane (Figure 3).

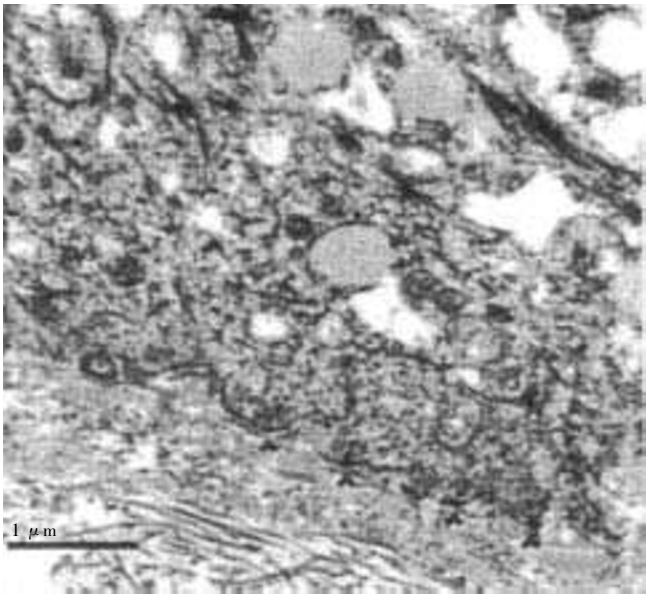

Figure 3. Transmission electron microscope image of rabbit oral mucosa epithelial cells after 3-week culture.

4. Discussion

Researches have showed that mouse fibroblasts has effective feeding effect on culture of various kinds of epithelial cells in vitro, which can effectively promote generation of epidermis, urinary tract epithelial cells or corneal endothelial cells, which further provides research direction for treatment of corneal endothelium diseases[18,19]. However, there has been no effective method to control heterogenous pathogen spreading risk caused by heterogenous cells, by now many countries have prohibited the clinical treatment of heterogenous tissue worldwidely[20]. Thus, exploration of inducing autologous fibroblasts to differentiate to corneal endothelial cells is quite essential.

At present, there are mainly two methods to treat feeder cells clinically including γ radiation and mitomycin C. The application of the former is gradually decreasing due to complicated operation and high cost. Different cells have different tolerance to mitomycin C[21-23]. Thus, before treating feeder cells we must first make sure the applicable concentration of mitomycin C. We found that as concentration of mitomycin C increased, cell proliferation rate and survival rate gradually decreased, indicating that normal physiological metabolism activity was significantly affected. To decrease residual of mitomycin C and effectively inhibit proliferation of fibroblast[24], we concluded that 5 μg/mL was the most appropriate mitomycin C concentration, which can maintain normal cellular metabolism and avoid nutrition loss of epithelial cell caused by cell over proliferation. Establishment of corneal endothelial cell needs seed cell, feeder cell and carrier. In our research, rabbit oral mucosa epithelial cell was used as seed cell, rabbit conjunctiva fibroblast was used as feeder cell and human denuded amniotic membrane was used as carrier to successfully establish tissue engineering corneal endothelium. The mechanism is as following: 1. Fibroblasts has the potential of multi differentiation, and repairment of epithelial cells are considered to be related to fibroblasts[25-28], thus, fibroblasts can differentiate to endothelial cells after induction; 2. Application of feeder cells and medium can promote cell growth and differentiation regulation of seed cells, which function significantly in controlling epidermal growth factor[29,30] to effectively promote migration and proliferation of seed cells; 3. human amniotic membrane was used as carrier to promote seed cell growth, and it has anti-inflammatory effect. The structure is simple and has no lymph tissue, which can reduce the risk of graft rejective reaction[31,32].

In conclusion, fibroblast cells have the potential of multi-directional differentiation, effective induction can promote emergence of intercellular desmosomes between seed cells and emergence of epithelial surface microvilli, and differentiate to the corneal endothelial cell. However, clinical application still needs more research and safety evaluation.

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