·Basic Research·

The study of human PDGF-B gene transferred to cat corneal endothelial cells

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Abstract

• AIM: To demonstrate that human platelet-derived growth factor-B (PDGF-B) cDNA could be expressed in primary cultured cat corneal endothelia cells by using gene transfer techniques; to explore a useful tool for the further studies of the molecular mechanisms of corneal endothelium failure and provide a potential effective genetic therapy for the blind patients.

• METHODS: Human PDGF-B cDNA was isolated from human placent by RT-PCR and inserted into $pcDNA_4$ vector to construct recombinant eukaryotic expression plasmid $pcDNA_4$-PDGF-B. The full length was confirmed by the DNA sequencing analysis. By tearing endothelium technique we obtained pure single layer of cat corneal endothelial cells. The $pcDNA_4$-PDGF-B eukaryotic expression vector was transferred into cat corneal endothelial cells by Effectene™ lipofectine. The transfection efficiency of Effectene™ lipofectine in $pcDNA_4$-B was detected with $pcDNA_4$-GFP. 5 days later, RT-PCR was used to check the PDGF-B expression. Cell viability was tested by modified tertrozalium salt (MTT) method. Cell morphology was observed under inverted phase contrast microscope.

• RESULTS: The human PDGF-B cDNA was isolated successfully from healthy parturien placent tissue and the sequence was confirmed by computer automatic sequence and PCR analysis. Pure single layer cat corneal endothelial cells were successfully cultured by tearing endothelium technique. Effectene™ lipofectine transfection technique could be effectively used to transfer $pcDNA_4$-PDGF-B into cat corneal endothelial cells *in vitro*, the transfection efficiency was 30%. RT-PCR result showed that human PDGF-B gene was highly expressed in transfected cat corneal endothelial cells. The expressed PDGF-BB protein promoted the viability of cat corneal endothelial cells.

• CONCLUSION: Human platelet-derived growth factor-B (PDGF-B) cDNA could be highly expressed in cultured cat corneal endothelial cells by gene transfection techniques. Expressed PDGF-BB protein significantly promoted the viability of cat corneal endothelial cells, thus provided a potential effective method for corneal endothelium blindness genetic therapy.

• KEYWORDS:platelet-derived growth factor;corneal endothelial cell; viability; gene transfection.

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INTRODUCTION

Corneal endothelial cells (CEC) of human being have limited proliferative ability after trauma, intraocular surgery and age-related injuries. The normal function of CEC is necessary for keeping corneal transparency [1]. In recent years some scholars tried to add growth factors to cell culture medium and resulted in the fast cultivation of CEC. However, these growth factors could work only for a limited period but not keep the CEC proliferation permanently. Therefore, we constructed eukaryotic expression vector $pcDNA_4$-PDGF-B, transferred it into in vitro cultured cat CEC to express biological active PDGF-BB protein, promote cell proliferation and viability in a high level for long term, so to provide an experimental foundation for corneal endothelium blindness genetic therapy study.

MATERIALS AND METHODS

Construction of eukaryotic expression vector $pcDNA_4$-PDGF-B Total human RNA was isolated from healthy parturien placent tissue according to the manual of Qiagen Rneasy Mini Kit. The purified RNA was analyzed by agrose gel electrophoresis and quantified spectraphotometrically.

PDGF-B cDNA was generated by RT-PCR using upstream primer containing BamH Ⅰ site and downstream primers containing Xho Ⅰ site. The sequences for the forward and reverse primers were as follows (5-3):5'CGCGGATCCA CCATGAATCGCTGCTGG3' (5-3):5'CCGCTCGAGCTA GGCTCCAAGGGTCTC3' according to the nucleic acid sequence encoding of human PDGF-B reported in GeneBank (NM_002608). The PDGF-B RT-PCR reaction mixture was firstly pre-denatured at 95℃ for 15 minutes, then amplified using 40 cycles, with one amplification cycle consist of 1 minute denaturation at 94℃, 1 minute primer annealing at 52℃, and 1 minute extension at 72℃. After sequencing the correct fragment were cut at BamH Ⅰ and EcoR Ⅰ sites with endonuclease and cloned directly into the expression vector $pcDNA_4$.

Primary culture of cat corneal endothelial cells Two months old cats, with no limit to sexuality, healthy without medical history were used in this study. Cats were anesthetized by Chloral Hydrate. 120 fresh eye balls were removed and immersed in D-Hanks solution with 100μ/mL penicillin and 100μg/mL phytomycin for 30min and then rinsed with sterile water, ruled out abnormality with examination. The corneas were excised under sterile condition and placed in a petri dish containing DMEM. Under a dissecting microscope, Descemet's membrane attached with endothelium was stripped from the stroma and placed in a 15-mL centrifuge tube containing 0.25% trypsin, incubated for 10 minutes at 37℃. Cells were detached by vigorous disruption with a flame-polished pipette, centrifuged and resuspended in culture medium DMEM with 0.5% fetal bovine serum then were incubated in tissue culture bottles at 37℃ in 5% CO_2 humidified atmosphere. Medium was changed every other day. Cells reached confluence in 10 to 14 days. Monolayer cultures of cat endothelial cells were harvested using 0.05% trypsin/0.02% EDTA solution.

Immunocytochemistry stainning of cat corneal endothelium cells Neuron specific enolase (NSE) is the specific mark protein of cat corneal endothelial cell, which could effectively distinguish endothelial cell from keratocyte. CEC could keep expressing NSE even after 20 passages while keratocyte never express NSE [2]. Immunocytochemistry stainning was performed to identify the corneal endothelium cells with anti-NSE antibodies. Briefly, 1×10^4 cells growing in chamber slides (Nalge Nunc International, Rochester, NY, USA) were fixed with 4% paraformaldehyde, rinsed with PBS and permeabilized with ice-cold acetone. Non-specific binding was blocked by incubating cells in 1% bovine serum albumin (BSA) for 30 minutes at room temperature. Added with anti-NSE antibodies (1:250 in PBS, Invitrogen Molecular Probes), cells were incubated overnight at 4(C then rinsed with PBS. The second antibody was then applied for 1 hour at room temperature. After rinsed with PBS, cells were applied with ABC elite and DAB. Rinsed with ddH_2O for 3 times, cells were then coverslipped with Geltol (Thermo Electron Corp., Waltham, MA, USA) as a mounting media and viewed under inverted phase contrast microscope.

Cell transfection CECs were divided into three groups: Normal CEC control group, CEC-$pcDNA_4$ control group and CEC-$pcDNA_4$-PDGF-B group. $2\text{-}8\times10^5$ primary cultured cells were subcultured into 60mm tissue culture plates at 37℃ in a 5% CO_2 humidified atmosphere.About 24 hours later, different medium was added to various groups separately.

Transfection efficiency assay Transfection efficiency was confirmed by detecting reporter gene. Reporter gene $pcDNA_4$-GFP was transferred into CEC in the same way. 48 hours later, counted the percentage of fluorescein stained cells in 10 fields randomly under inverted phase contrast microscope to confirm the transfection efficiency.

PDGF -B gene expression assay Five days after transfection, CEC total RNA in three groups was isolated by Trizol reagent. The purified RNA was analyzed by agrose gel electrophoresis and quantified spectraphotometrically. PDGF-B expression fragment was extracted by RT-PCR with β-actin as endo-reference, with forward primer 5'ACCCCCACTGAAAAAGATGA3' and reverse primers 5'ATCTTCAAACCTCCATGATG3'. The PDGF-B RT-PCR reaction mixture was firstly pre-denatured at 95℃ for 15 minutes, then amplified using 10 cycles, with one amplification cycle consisting of 1 minute denaturation at 94℃, 1 minute primer annealing at 56℃, and 1 minute extension at 72℃.

Cell viability assay Cell viability was tested by modified MTT method. The biological activity of expressed PDGF-BB was determined based on the fact that PDGF-BB can promote the differentiation and viability of cat corneal endothelial cells. 5 days after being transfected, CECs were subcultured in 5.0×10^4/mL to 96-well tissue culture plate for another 48 hours. For MTT assay, cells were switched to MTT solutions (5mg/mL) 20μL, 37℃, 5% CO_2 for 4 hours, then solution was discarded and 150μL DMSO was added to each well, traced blender shock for 10 minutes, then detected the OD value in 490nm.

Morphology observation The CECs proliferation status in three groups were evaluated under inverted phase contrast microscope since transfection.

Statistical Analysis The MTT results were analyzed using software SPSS 11.0. The mean (Mean±SD) was calculated and *t* test was performed in accordance with different density and time points of the results.

RESULTS

Construction of $pcDNA_4$-PDGF-B vector In this study, 725 bp PDGF-B cDNA was isolated successfully from

human placent tissue by RT-PCR. The sequence of the amplified PDGF-B was the same as the human PDGF-B described in GENEBANK (NM_002608). Mature fragment was cloned into the expression vector, PDGF-B expression plasmid pcDNA$_4$-PDGF-B was constructed successfully (Figure 1).

Cell morphology observation Primary cultured CECs adhered in 24 hours, 1-2 weeks later expanded into massive single layer cells in shapes of similar circular and polygon. Cells were passaged and inoculated in 96-well board, 24 hours later most of them adhered.

Immunocytochemistry stainning of cat CEC NSE staining showed buffy macrobead could be found in the cytoplasm of corneal endothelium cells with over 98% positive rate(Figure 2A), while it was negative in keratocyte control group (Figure 2B).

Transfection efficiency Transfection efficiency was confirmed by detecting report gene. 48 hours after reporter gene pcDNA$_4$-GFP being transfered to CEC, counted the percentage of fluorescein stained cells in 10 fields under inverted phase contrast microscope. Results showed that 30% of CEC cells appearing GFP positive. (Figure 3).

Expression of PDGF-B in CEC cells Five days after transfection,725bp PDGF-B cDNA was isolated successfully in all three groups. The quantities of PDGF-B cDNA were in the same moderate level in two control groups, while that was much higher in CEC-pcDNA$_4$-PDGF-B group. Semiquantitative analysis of RT-PCR products of three groups was shown in Table 1 and Figure 4.

Biological activities of the expressed PDGF-B In this study, the bioactivity of expressed PDGF-BB was detected based on the fact that it could promote CEC cells differentiation and viability. The MTT results showed 5 days after transfection, the expressed PDGF-BB could obviously promote viability of cat endothelial cells(P<0.01) vs the two control groups. Two control groups showed no significant differences(P>0.05) in between. Blank plasmid pcDNA$_4$ did not change the viability of CEC and there was no toxic effect of liposome to CEC (Figure 5) .

Morphologic changes of CEC after transfection Cells in CEC-pcDNA$_4$-PDGF-B group proliferated faster obviously compared to the two control groups. After the first 24 hours, cells in all the three groups adhered in shapes of roundness and polygon. However, 5 days later, cells in CEC-pcDNA$_4$-PDGF-B group proliferated into bigger scales in shapes of regular triangle to hexagon with distinct boundary(Figure 6), while the number of cells was significantly lesser in two control groups(Figure 7,8). In the same time, CEC-pcDNA$_4$-PDGF-B group for 5 days was NSE staining positive which showed the character of CEC did not changed.

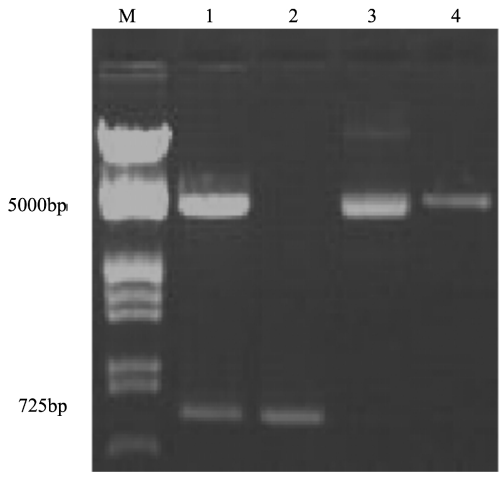

Figure 1 gel electrophoresis of pcDNA$_4$-PDGF-B RT-PCR products M: Λ DNA/Hind Ⅲ +EcoR Ⅰ PCR marker; 1: recombinant pcDNA$_4$-PDGF-B; 2: PDGF-B cDNA; 3,4: plasmid pcDNA$_4$.

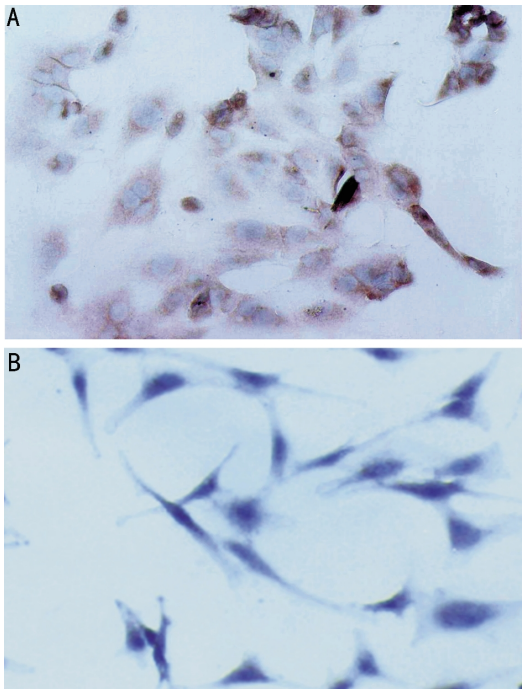

Figure 2 NSE stainning of cat CEC and keratocyte A: NSE staining positive in CEC (×400); B: NSE staining negative in keratocyte (×400).

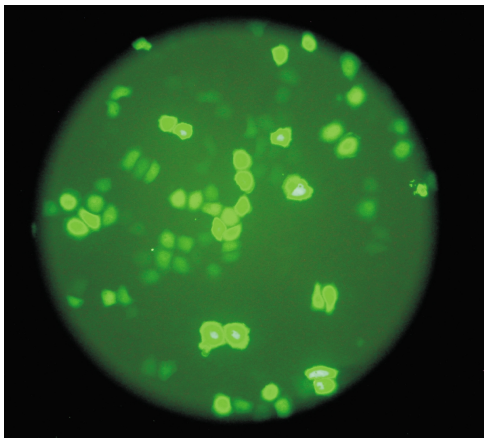

Figure 3 Reporter gene GFP expressed in fluorescein stained cells(×100).

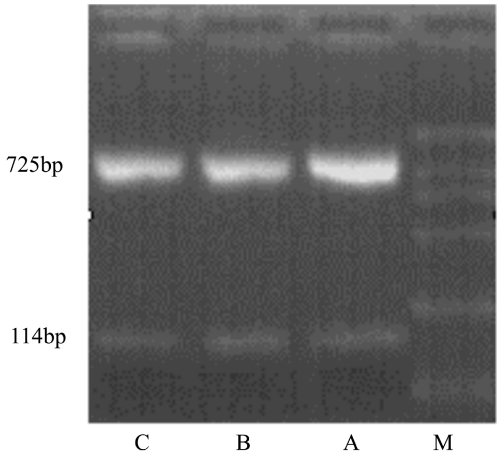

Figure 4 Semiquantitative analysis of RT-PCR products M: DL2000 marker; A: CEC-pcDNA$_4$-PDGF-B group; B: Normal CEC control group; C: CEC-pcDNA$_4$ control group.

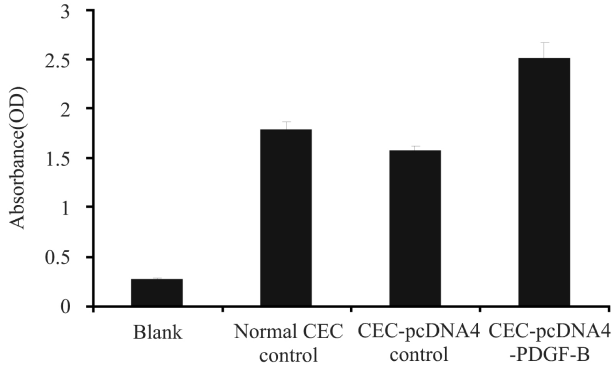

Figure 5 MTT result of four groups (A value) PS: CEC-pcDNA$_4$- PDGF-B group showed difference compared with other three groups (P<0.01); while Normal CEC control group showed no difference compared with CEC-pcDNA$_4$ control group (P> 0.05).

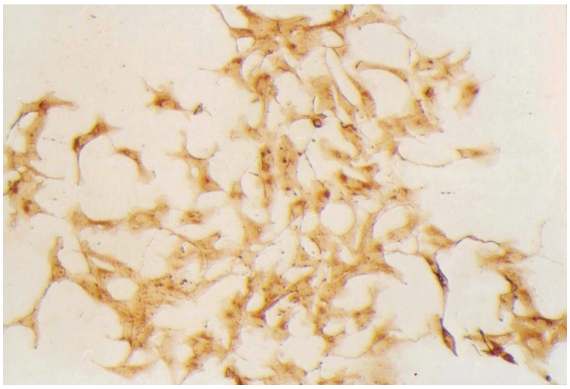

Figure 6 CEC -pcDNA$_4$ -PDGF -B group 5 days after transfection NSE staining positive (×200).

Table 1 Semiquantitative analysis of RT-PCR products in three groups

Product quantity	PDGF-B (725bp)	β-actin (114bp)	PDGF-B/β-actin
A	45	10	4.5
B	33	9	3.67
C	31	9	3.44

Groups: A: CEC-pcDNA$_4$-PDGF-B group B: normal CEC control group C: CEC-pcDNA$_4$ control group.

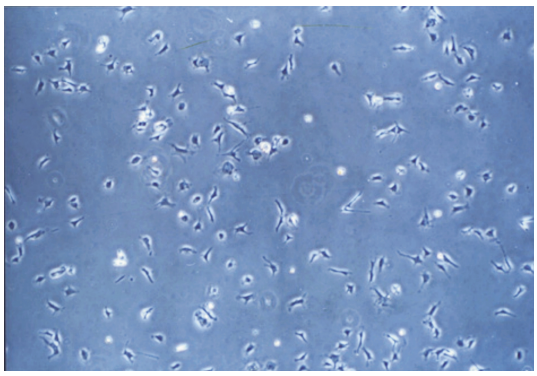

Figure 7 Normal CEC control group 5 days after transfection (×200).

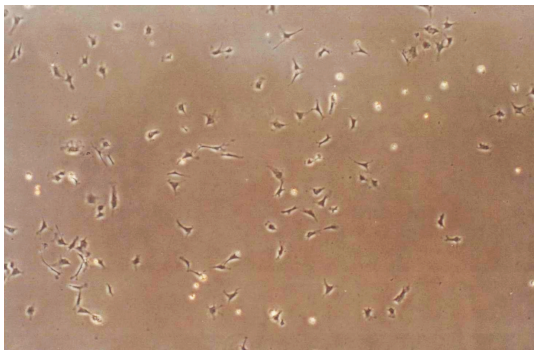

Figure 8 CEC-pcDNA$_4$ control group 5 days after transfection (×200).

DISCUSSION

PDGF was first discovered by Ross *et al* [3] in 1974 in the platelet, so it was called plated derived growth factor. PDGF protein is a dipolymer of A,B integrated by disulfide linkage, which has three forms named PDGF-AA,PDGF-BB and PDGF-AB with the relatively molecular mass 30000[4,5]. Among these three forms, PDGF-B is primarily expressed in endothelial cells. PDGF can potentially promote the mitogen of various connective tissue cells, not only promote cell growth but also provoke the recombination of cystoskeleton and cell differentiation. PDGF-B probably has significant effect in promoting the proliferation and repair in CEC trauma, which shows great value in basic research and clinical application of CEC blindness treatment.

As a paracrine growth factor, PDGF can regulate the affluxion of Ca^{2+} in CEC, change the cystoskeleton of CEC and corneal epithelial cells, promote the cytodieresis of CEC and corneal cells [6-8], facilitate the trauma repair of CEC, keratocyte and corneal epithelial cell [7]. However, the expression of PDGF is strictly controlled and can not be detected in CEC and anterior chamber in normal condition. Therefore, in this study we extracted total RNA from human placent tissue, isolated PDGF-B cDNA by RT-PCR and constructed the eukaryotic expression vector pcDNA$_4$-PDGF-B. The result of sequencing showed a 725bp cDNA identical with the human PDGF-B described in GENEBANK(NM_002608).

Transgene technique can effectively change cells bionomics in somatocyte level. The key of transgene technique is to choose a suitable vector according to different treatment requirement of diseases. An ideal vector should transfer the aimed gene into target tissues and express safely, effectively and stably. Some scholars had tried to use vectors such as particle gun, cationic polymerization and adenovirus [9-13], however these vectors have various weakness such as transient expression, need to repeat application, induce the immune reaction of organism, wild type viral infection and so on. These weaknesses have greatly circumscribed their application. Therefore, we applied the classic liposome vector in this study. We transferred recombinant eukaryotic expression vector $pcDNA_4$-PDGF-B into cultured second generation cat CEC and the efficiency is 30%. Although its efficiency is not as high as the virus vector [13], the liposome has no protein element in it, which greatly decreased the inflammatory reaction so is more safe and effective than virus vector. It has many superiorities such as quickly expressing exogenous gene, transferrable to cells in undividing phase, and unconformable to the genome of target cells, without mutation and cancerigenic hazard. So theoretically it is more fit for the treatment of trauma repair. Thus liposome is frequently adopted by scholars nowadays[14]. The liposome vector will show more safety and benefits in the next step clinical research.

There are three groups in this study: normal CEC control group, CEC-$pcDNA_4$ control group and CEC-$pcDNA_4$-PDGF-B group. 5 days after transfection, same quantity of cells in the three groups were harvested, total RNA was extracted and the expression of PDGF-B cDNA was detected by RT-PCR. Statistical result showed the expression level of PDGF-B was much higher in CEC-$pcDNA_4$-PDGF-B group than the two control groups, which makes it possible to promote CEC proliferation and differentiation. Liposome shows no toxic effect in transfection.

MTT technique can be used to detect cell viability. The result of this study showed manifest improvement of viability in CEC-$pcDNA_4$-PDGF-B group, while there was no significant difference in the two control groups. This means liposome shows no toxic effect to the target cells. The reason perhaps lies in that PDGF-B gene expressed protein in target cells and secreted it out of the cells by autocrine and paracrie action. Then the expressed protein reacted with cell surface receptors, showed specific biologic activity and promoted the viability and differentiation of *in vitro* cultured cat CEC.

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