

Effect of recombinant human platelet –derived growth factor B on cat corneal endothelial cell viability mediated by adeno–associated virus

Wen-Juan Luo¹, Xiao-Ming Xing², Chuan-Fu Wang¹, Li-Ting Hu¹, Gui-Qiu Zhao¹, Xiang-Ping Liu³, Kun Wu¹, Hui Li¹

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¹Department of Ophthalmology, the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China

²Department of Pathology, the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China

³Central Laboratory of the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China

Correspondence to: Wen-Juan Luo. Department of Ophthalmology, the Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China. Luowj76@gmail.com

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Abstract

• **AIM:** To transduce recombinant human platelet-derived growth factor B (PDGF-B) gene adeno-associated virus(AAV) to *in vitro* cultured cat corneal endothelial cell (CEC) and observe the effect of the expressed PDGF-BB protein on the viability of cat CEC.

• **METHODS:** Cat cornea endothelium was torn under microscope and rapidly cultivated in DMEM to form single layer CEC and the passage 2 endothelial cells were used in this study. The recombinant human PDGF-B gene AAV was constructed and transduced into cat CEC directly. Three groups were as following: blank control group, AAV control group and recombinant AAV group. At 24 hours, 48 hours, and 5 days after transduction, total RNA was extracted from the CEC by Trizol and the expression of PDGF-B gene was detected by fluorescence quantitative polymerase chain reaction. Viability of the transduced CEC was detected at 48 hours after transduction by MTT assay. Cell morphology was observed under inverted phase contrast microscope.

• **RESULTS:** With the torn endothelium culture technique, we rapidly got single layer cat CEC. At 24 hours, 48 hours and 5 days after transduction, fluorescence quantitative polymerase

chain reaction showed there was no significant difference of the expressed PDGF-B gene mRNA between blank control group and AAV control group ($P>0.05$). In contrast, there were significant differences between two control groups and recombinant AAV group ($P<0.05$). MTT assay showed that in recombinant AAV group, the expressed PDGF-BB protein could promote the viability of cat CEC. Morphology observation showed at 48 hours after transduction, cells in CEC-AAV-PDGF-B group proliferated into bigger scales in regular triangle to hexagon shape with distinct boundary, while the number of cells was significantly less in the two control groups.

• **CONCLUSION:** The recombinant AAV-PDGF-B expresses biological active PDGF-BB protein in cat CEC, which promotes the viability and proliferation of cells.

• **KEYWORDS:** platelet-derived growth factor; corneal endothelial cell; transduction; viability; proliferation

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INTRODUCTION

Corneal endothelial cell (CEC) is the key factor to maintain transparency of cornea. Once there is derangement or trauma in CEC, it will lead to edema of cornea and partial or total deprivation of corneal transparency^[1,2]. Being deficient of mitosis in human CEC, it is hard for cornea to repair automatically after trauma and intraocular operation.

Human platelet-derived growth factor B (PDGF-B) can potentially promote the mitosis in various connective tissue cells, not only promote the recombination of cytoskeleton but also provoke cell differentiation^[3]. PDGF can change the cytoskeleton of CEC and corneal epithelial cells, promote the cytodieresis of CEC and corneal stroma cells^[4,5], so to facilitate corneal trauma repair. PDGF-B protein could be

added directly to *in vitro* cultured cell or trauma tissue. However, the growth factor produced by biological refining or genetic engineering is not only expensive but also easily being inactivated by protease on trauma surface. So that greatly limits its clinical practicability.

In this study, we transduced recombinant PDGF-B gene adeno-associated virus (AAV) to cat CEC, let it express biological active PDGF-BB protein to promote the viability and proliferation of CEC and so to lay a basis for genetic treatment of corneal disease.

MATERIALS AND METHODS

Materials

Design and synthesize of primers Specifically designed probe primers and fluorescent probes with ABI Primer Express software and analyzed with BLAST. 1) human PDGF-B gene: the forward and reverse primers were as following 5'-CTC GATCCG CTC CTT TGA TG-3', 5'-GTC TTGCAC TCG GCG ATC AT -3'. Fluorescent probes: 5'-FAM- CCTGAA CAT GAC CCG CTC CCA CTC T -TAMRA-3', the length of the target gene is 189bp; 2) cat glyceraldehyde-3-phosphate dehydrogenase (endo-reference): the forward and reverse primers were as following 5'- TCA AGG CTG AGA ACG GGA AAC -3', 5'- AGC CTT CTC CAT GGT GGT GAA -3'. Fluorescent probes: 5'-FAM-CAG GAG CGA GAT CCC GCCAAC AT -TAMRA-3', the length of the target gene is 146bp.

Methods

Primary culture of cat CEC Healthy 2 months old cats, weighing 250g-300g, without sex restriction. A total of 120 eye balls, ruled out abnormality with examination. Cats were anesthetized by Chloral Hydrate. Fresh cat eyes were moved out and immersed in D-Hanks solution added 100U/mL penicillin and 100µg/mL phytomycin for 30 minutes and then rinsed with sterile water. The corneas were excised under sterile conditions and placed in a petri dish containing DMEM. Under a dissecting microscope, Descemet's membrane with the attached endothelium was stripped from the stroma and placed in a 15mL centrifuge tube containing 0.25% trypsin, incubated for 10 minutes at 37°C. Cells were detached by vigorous disruption with a flame-polished pipette, were centrifuged and resuspended in culture medium DMEM containing 0.5% fetal bovine serum then were incubated in tissue culture bottles at 37°C in a 5% CO₂ humidified atmosphere. Medium was changed every other day. Cells reached confluence in 10-14 days. Monolayer cultures of cat endothelial cells were harvested using 0.05% trypsin or 0.02% EDTA solution. The passage 2 endothelial cells were used in this experiment.

Immunocytochemistry staining of cat CEC Neurone specific enolase (NSE) is the specific mark protein of corneal CEC, which could effectively distinguish CEC from keratocyte. CEC could keep expressing NSE even after 20

passages, while keratocyte never express NSE^[9]. Here immunocytochemistry staining by anti-NSE antibody was performed to identify the CEC. Briefly, 1×10⁴ 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. For visualization of NSE, anti-NSE antibody (1:250 in PBS, Invitrogen Molecular Probes) was applied, cells were incubated overnight at 4°C, then rinsed with PBS. Then the second antibody was applied followed by incubating for 1 hour at room temperature. After being rinsed with PBS for 4 times, CEC was applied with ABC elite for 30 minutes followed by rinsing with PBS for 3 times and being applied DAB for 5-10 minutes. Being rinsed with ddH₂O for 3 times, CEC was then coverslipped with Geltol (Thermo Electron Corp., Waltham, MA, USA) as a mounting media and viewed using inverted phase contrast microscope.

CEC transduction There were three groups in the study: normal CEC control group; CEC-AAV control group and CEC-AAV-PDGF-B group. The AAV was diluted with DMEM to 1×10⁹ in titres, discard the supernatant liquid 4 hours later, and then was applied to DMEM with 10% blood serum. All the cells were harvested after transduction for 24 hours, 48 hours and 5 days.

PDGF-B gene expression assay Total RNA in three groups was isolated by Trizol reagent. 1)The purified RNA was analyzed by agrose gel electrophoresis and quantified spectrophotometrically. The PDGF-B cDNA was synthesized in 10µL RT-PCR reaction mixture: 5×ExScript™ Buffer 2µL, 10mmol/L dNTP Mixture 0.5µL, 100µmol/L Random 6mers 0.5µL, ExScript™ Rtase (200U/µL) 0.25µL, RNase Inhibitor (40U/µL) 0.25µL, total RNA 0.5µg. Reverse transcription at 42°C for 12 minutes, then reverse transcriptase was deactivated at 95°C for 2 minutes; 2) Synthesize with specific primers: specifically designed probe primers of human PDGF-B gene and cat glyceraldehyde-3-phosphate dehydrogenase were applied to PCR reaction. The reaction mixture was pre-denatured at 95°C for 10 seconds, denatured at 95°C for 5 seconds, and extended at 60°C for 45 seconds, amplified in 40 cycles; 3) Fluorescent quantitative PCR reaction: the reaction mixture was 20µL including 2×Premix ExTaq™ buffer 10µL, primers concentration 0.2µmol/L, probes concentration 0.05µmol/L, 50×ROX Reference Dye II 0.4µL, cDNA 2µL. The reaction mixture was pre-denatured at 95°C for 10 seconds, denatured at 95°C for 5 seconds, and extended at 60°C for 45 seconds, amplified in 40 cycles. The mean Ct value of the two values in each group was analyzed by 2^{-ΔΔCt} to get the relative expressing quantity. $\Delta\Delta Ct = (Ct_{\text{target gene}} -$

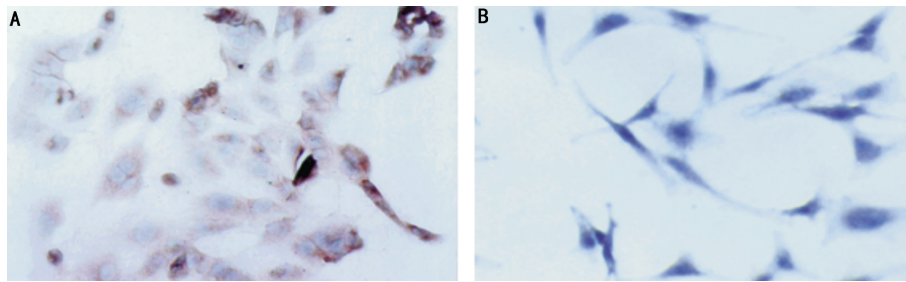


Figure 1 NSE staining of cat CEC and keratocytes A: NSE staining was positive in cat CECs ($\times 400$); B: Keratocytes had no specific antigen staining ($\times 400$).

$Ct_{(endo-reference\ gene)}^{transduced} - (Ct_{target\ gene} - Ct_{endo-reference\ gene})_{control}$. Here the CT value means the cycles for the reaction mixture to reach the fluorescent threshold in PCR reaction.

Cell viability assay Cell viability was tested by modified MTT method. Forty-eight hours after transduction, CEC in three groups were subcultured in $5.0 \times 10^4/mL$ to 96-well tissue culture plate for another 24 hours. For MTT assay, cells were switched to MTT solutions (5mg/mL) $20\mu L$, $37^\circ C$, 5% CO_2 for 4 hours, then solution was discarded and $150\mu L$ DMSO was added to each well, traced blender shock for 10 minutes, then detected the OD value in 490nm.

Morphology observation Kept observing the cells growth condition under inverted phase contrast microscope after transduction, evaluated the differences of CEC proliferation in three groups.

Statistical Analysis The MTT results were analyzed using software SPSS 11.5. The mean \pm SD was calculated and t test was performed in accordance with different time points of the results.

RESULTS

Primary Cultured Cell Morphology Observation CEC adhered in 24 hours primary culture, two weeks later expanded to 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 Staining of Cat CEC NSE staining showed buffy macrobead could be found in the cytoplasm of corneal endothelium cells with over 98% positive rateed (Figure 1A), while it was negative in keratocyte control group (Figure 1B).

Synthesize with Specific Primers The total RNA of all three groups is 1.8-2.0 in A_{260}/A_{280} . The electrophoresis result of PCR product showed two lines in 189bp and 146bp, which consisted with human PDGF-B gene and cat glyceraldehyde-3-phosphate dehydrogenase gene (Figure 2). The sequencing result showed the 189bp line was human PDGF-B gene which totally consisted with genebank.

Fluorescent Quantitative PCR Reaction The results of fluorescent quantitative PCR reaction in every group got classic S shape curve. The Ct value of glyceraldehyde-3-

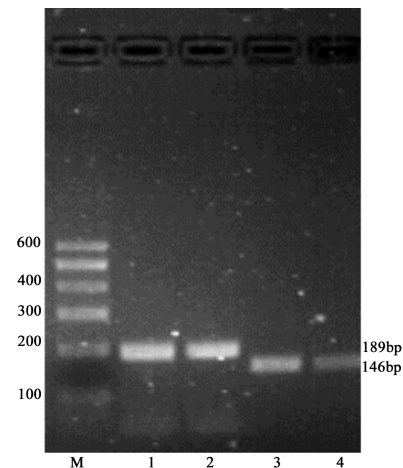


Figure 2 Agarose gel electrophoresis of PCR products of target genes M: 100-600bp DNA marker; 1,2: Human PDGF-B gene; 3,4: Cat glyceraldehyde-3-phosphate dehydrogenase gene.

phosphate dehydrogenase diversified trifle which meant the initiate quantity of templates in each group was almost the same. At 24 hours, 48 hours and 5 days after transduction, the human PDGF-B mRNA detected by fluorescence quantitative PCR showed there was no significant difference between normal CEC control group and CEC-AAV control group ($P > 0.05$). There was significant differences between the two control groups and CEC-AAV-PDGF-B group ($P < 0.05$). The quantity of human PDGF-B mRNA expressed in CEC-AAV-PDGF-B group showed no significant differences among 24 hours, 48 hours and 5 days (Figure 3).

Cell Viability Tested by Modified MTT Method Forty-eight hours after transduction, the A_{490} in blank control group, normal CEC control group, CEC-AAV control group and CEC-AAV-PDGF-B group were 0.26 ± 0.01 , 1.58 ± 0.16 , 1.63 ± 0.17 and 1.81 ± 0.20 (Figure 4).

Compared with blank control group, normal CEC control group and CEC-AAV control group, the viability activity in CEC-AAV-PDGF-B group improved significantly ($P < 0.05$). There was no significant difference between normal CEC control group and CEC-AAV control group ($P > 0.05$).

Morphologic Changes of CEC After Transduction Cells in CEC-AAV-PDGF-B group proliferated obviously

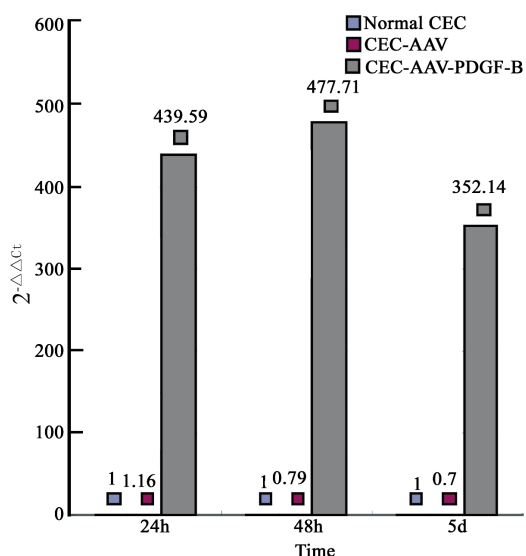


Figure 3 Relative quantitative analysis of human PDGF-B.

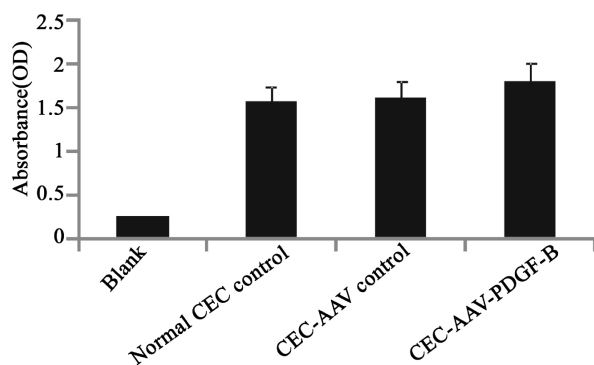


Figure 4 MTT result of four groups (A value).

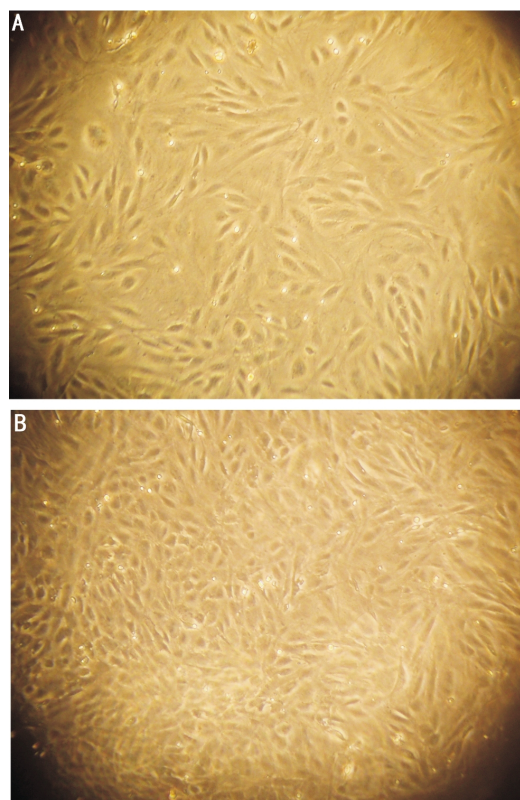


Figure 5 Cells cultured for 48 hours after PDGF -B transduction (×200) A: Normal CEC control group; B: CEC-AAV-PDGF-B group.

faster than the control groups. After the first 24-hour, cells in all three groups adhered in shapes of roundness and polygon. However, 48 hours later, cells in CEC-AAV-PDGF-B group proliferated into bigger scales in regular triangle to hexagon shape with distinct boundary, while the number of cells was significantly less in the control groups(Figure 5).

DISCUSSION

Transgene technique can effectively change cells bionomics in somatocyte level. The key of genetic therapy is to transduce and express the aim gene into target tissues safely, effectively and stably. Liposome is the mostly used non-virus vectors nowadays, it has high safety, high transient expression, but low transduction efficiency. The AAV particle is easy to permeate cell external shield, the aim gene is easy to integrate into the DNA of host cells to stably express in long-term [6]. However, it could not duplicate in passage cells but had to be packaged repeatedly. The produce of new adeno-associated virus particle need to be assisted by helper virus and package virus, or else it could only infect latently [7].

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 [8,10]. Among these three forms, PDGF-BB is primarily expressed in endothelial cells. In this study, we used AAV as the vector of human PDGF-B gene [11]. The recombinant human AAV-PDGF-B was transduced into *in vitro* cultured cat CEC directly. The result of fluorescence quantitative polymerase chain reaction showed high and stable mRNA expression of the human PDGF-B gene. Due to not being purified, the recombinant human AAV-PDGF-B is in low titres, however high express of the PDGF-B mRNA was detected in the study. In the same time, recombinant human AAV-PDGF-B could be transduced directly to target cells, therefore, more available in the *in vivo* study. Results of MTT and morphological observation showed recombinant AAV-PDGF-B expressed biological active PDGF-BB protein and notably promoted the viability and proliferation of CEC. So there will be great clinical significance for application of recombinant human AAV-PDGF-B in the treatment of corneal blindness.

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