Cloning, expression and functional analyses of human platelet-derived growth factor-B chain peptide for wound repair of cat corneal endothelial cells

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Objective: To investigate the biological function of platelet-derived growth factor B (PDGF-B) on the survival and proliferation of cat corneal endothelial cells so as to provide bases for further studies of its role in wound repair and its clinical application.

Methods: Total RNA was extracted from the placenta tissues of healthy pregnant women undergoing hysterotomy and PDGF cDNA was obtained with reverse transcription-polymerase chain reaction (RT-PCR). The prokaryotic expression vector pET-PDGF-B was constructed and expressed the recombinant PDGF-B in Escherichia coli (E.coli) BL21 (DE3). After purification and refolding on Ni²⁺-chelation affinity chromatography (NTA) column, it was used to culture cat corneal endothelial cells. Cell proliferation was tested by modified tetrazolium salt (MTT) and flow cytometer. And the morphologic change and the ultrastructure were observed under an inverted phase contrast microscope, a scanning electron microscope and a transmission electron microscope, respectively.

Results: PDGF-B chain peptide (PDGF-BB) gene was successfully inserted into the prokaryotic expression vector, pET-28a (+). The purified recombinant protein pET-PDGF-B showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the molecular weight of about 27 u, which was in agreement with the deduced value. MTT and flow cytometry showed that PDGF-BB promoted the survival and proliferation of cat corneal endothelial cells.

Conclusions: The construction of recombinant prokaryotic expression vector pET-PDGF-B and the preparation of PDGF-BB protein provide a foundation for further study of the function of PDGF-BB and producing biological PDGF-BB protein. The expressed PDGF-BB promotes the proliferation of cultured cat corneal endothelial cells.

Key words: Platelet derived growth factor; Gene expression; Corneal endothelium; Proliferation

Platelet-derived growth factor (PDGF) was first discovered by Ross et al in 1974 and it was so named for it was firstly purified from platelets. However, many other cells, such as endothelial cells, macrophages and smooth muscle cells, are found to produce and secrete PDGF or PDGF-like growth factors. PDGF is a potential mitogen of many cells, including fibroblasts, smooth muscle cells and endothelial cells. PDGF can increase cell proliferation and differentiation and stimulate cytoskeleton recombination. PDGF-BB may play an important role in the mitosis and trauma repair in corneal endothelial cells. In this study, to determine the effect of PDGF-BB on corneal endothelial cells, prokaryotic expression vector pET-PDGF-B was constructed. The recombinant PDGF-B proteins expressed in Escherichia coli (E.coli) bacteria (strain BL21), purified and refolded on Ni²⁺-chelation affinity chromatography (NTA) column. And then they were applied to cat corneal endothelial cells cultured in vitro. The proliferation and differentiation of the cells were studied to provide experimental data for clinical application.
METHODS

Materials
Restriction endonucleases and T4 DNA ligase were purchased from Promega (USA). Omniscript reverse transcriptase kits, mini-MTM DNA extraction system and gel extraction mini prep kit were purchased from Qiagen Company, USA. Pyrobest DNA polymerase was obtained from Takara Biotechnology Co. Ltd (Dalian, China). Bacterial expression plasmid pET28a(+) and E. coli bacteria (strain BL21 and strain TG1) were preserved in our own lab. Histidine bind kits were obtained from Novagen Company (USA). Reagents for protein refolding were purchased from Calbiochem (Dalian, China). The polylysine, fetal calf serum (FCS), horse serum and Dulbecco modified Eagle medium (DMEM) were purchased from Gibco (USA).

Construction of pET-PDGF-B expression vector
Total human RNA was isolated from the placenta tissues of healthy pregnant women undergoing hysterotokotomy according to the instructions of Qiagen Rn ease mini kits. The purified RNA was analyzed by agarose gel electrophoresis and quantified spectrophotometrically. PDGF-B mature fragments were generated by reverse transcription-polymerase chain reaction (RT-PCR) using upstream primers containing BamH I site and downstream primers containing EcoR I site. The sequences for the forward and reverse primers were as follows (5'-3'): gc ggtacc atg aat cgc tgc tgg gcg ctc and (5'-3') gc gaatcc tca ggc tcc aag ggt ctc ctt, according to the nucleic acid sequence encoding human PDGF-B reported in the Genebank (NM_002608). The PDGF-B PCR reaction mixture was firstly pre-denatured at 95°C for 15 minutes, then amplified for 40 cycles, with one amplification cycle consisting of denaturation for 1 minute at 94°C, primer annealing for 1 minute at 52°C, and extension for 1 minute at 72°C. The PCR products were cloned into plasmid pBluescript SK(+) (pBSK) vector by T4 ligase and amplified by plasmid extract kits. After sequencing, the correct fragments were cut at the BamH I and EcoR I sites and cloned directly into the expression vector pET28a (+) under the control of T7 promoter (Fig.1).

Expression and purification of human PDGF-B
One of our main aims of this study was to obtain recombinant human PDGF-BB protein. E. coli bacteria (strain BL21) were transformed with expression vector pET28a containing mature fragments of human PDGF-B cDNAs and grown at 37°C till an A600 being 0.8 approximately. The bacteria were induced by 1.0 mmol/L isopropyl-beta-D-thiogalactoside (IPTG). After 5 hours, the bacteria were harvested and the sample was sonicated in a tube on ice until the sample was no longer viscous. The recombinant proteins formed inclusion bodies and the inclusion bodies were collected and suspended in 20 ml wash buffer (2 mol/L urea, 0.5 mol/L NaCl, 10% glycerol, 1% TritonX-100, and 50 mmol/L 2-mercaptoethanol, pH 8.2) per 100 ml culture media. This step was repeated for several times to release more trapped proteins. The supernatant was removed from the final centrifugation and the final pellets of purified inclusion bodies were dissolved in 5 ml dissolution buffer (6 mol/L urea, 0.5 mol/L NaCl, 10% glycerol, 1% TritonX-100, 20 mmol/L Tris, pH 10.5) per 100 ml culture media. Since six consecutive histidine residues were expressed at the N-terminal end of the proteins, the proteins were purified and refolded on Ni2+-NTA column, namely, single-step purification and refolding method. The dissolved supernatant was filtered through a 0.45-micron membrane and then loaded onto a Ni2+-NTA column. After washed off the protein contaminants with 10 col-
Primary culture of cat corneal endothelial cells

Cats were anesthetized excessively by Chloral Hydride and fresh cat eyes were moved out and immersed in D-Hanks solution containing 100 U/ml penicillin and 100 μg/ml streptomycin 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, the membrane of the posterior elastic layer with the attached endothelium was stripped from the stroma and placed in a 15-ml centrifuge tube containing 0.25% trypsin. The tissues were incubated for 10 minutes at 37°C and then the cells were detached by vigorous disruption with a flame-polished pipette. The cells were centrifugated (1000 r/min) and resuspended in culture medium of DMEM. Under a dissecting microscope, the membrane of the posterior elastic layer with the attached endothelium was stripped from the stroma and placed in a 15-ml centrifuge tube containing 0.25% trypsin. The tissues were incubated for 10 minutes at 37°C and then the cells were detached by vigorous disruption with a flame-polished pipette. The cells were centrifugated (1000 r/min) and resuspended in culture medium of DMEM containing 0.5% fetal bovine serum. Then the cells were incubated in tissue culture bottles at 37°C in a 5% CO₂ humidified atmosphere, cat corneal endothelial cells by monolayer changed every other day. The cells reached confluence in 10-14 days. The cat endothelial cells by monolayer culture were harvested with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution.

Immunocytochemical staining of cat corneal endothelial cells

Neurone specific enolase (NSE) is a specific marker protein of cat corneal endothelium, which could effectively distinguish endothelial cells and keratocytes. In this study, immunocytochemical staining with anti-NSE antibodies was performed to identify the corneal endothelial cells. Briefly, 1×10⁶ cells grew in the chamber slides (Nalge Nunc International, Rochester, New York, USA), which were fixed with 4% paraformaldehyde, rinsed with phosphate buffered saline (PBS) and permeabilized with ice-cold acetone. Non-specific binding was blocked through incubating the cells in 1% bovine serum albumin (BSA) for 30 minutes at room temperature. For visualization of NSE, anti-NSE antibodies (1:250 in PBS, Invitrogen Molecular Probes, USA) were added and the cells were incubated overnight at 4°C and then rinsed by PBS. Then second antibody (biotinylated goat anti-rabbit IgG) was added and the cells were incubated for 1 hour at room temperature and rinsed by PBS for 4 times. Avidin-biotin-peroxidase complex (ABC) elite was added and the cells were incubated for 30 minutes and rinsed by PBS for 3 times. Diaminobenzidine (DAB) was added to develop the coloring for 5-10 minutes. After rinsed by double distilled water(ddH₂O) for 3 times, the cells were then cover-slipped with Geltoil (Thermo Electron Corp, Waltham, MA, USA) as a mounting media and observed under the inverted phase contrast microscope.

Biological activity assay

The cells of 1×10⁴-1×10⁵ in number were suspended in 0.2 ml DMEM containing 0.5% fetal bovine serum and subcultured into 96-well tissue culture plates at 37°C in a 5% CO₂ humidified atmosphere. The biological activities of PDGF-BB were determined based on the fact that it can promote the differentiation and proliferation of cat corneal endothelial cells. Modified tertrozalium salt (MTT) method was used to assay the proliferation of cells. About 24 hours later, the medium was discarded and conditional media were added in various groups after cell attachment. In details, at 37°C in a 5% CO₂ humidified atmosphere, cat corneal endothelial cells in the control group were cultured in DMEM, while the cells in the experimental groups were exposed to DMEM plus 1,10, 25, 50, 75, and 100 ng/ml PDGF-B, respectively. Seven days later, the effects of PDGF-BB on cell survival and proliferation were determined by MTT and flow cytometric analysis. For MTT assay, the cells were transferred to 20 μl MTT solution (5 mg/ml) at 37°C in a 5% CO₂ humidified atmosphere. And 4 hours later, the solution was discarded and 150 μl dimethyl sulfoxide (DMSO) was added in each well. After being shaked in a micro-blender for 10 minutes, the optical density (OD) value was detected in 490 nm.
Then the growth conditions of the cells were observed under the inverted phase contrast microscope periodically.

**Flow cytometric analysis of cell proliferation**

About $1 \times 10^6$ corneal endothelial cells treated with or without 100 ng/ml recombinant PDGF-B for 7 days were collected with Puck’s EDTA at 37°C and then washed in PBS containing 1 mmol/L EDTA (PBS/EDTA). The cells were fixed in 700 μl high grade Ethanol (EtOH, 100%) at -20°C and the tube was inverted for several times to ensure mixing properly and a good fixing of the cells. Then the cells were washed for 2-3 times with PBS/EDTA, resuspended in 1 ml DNA staining solution (2.5 μg/ml propidium iodide and 0.5 mg/ml RNase A in PBS), put into 5 ml fluorescence activated cell sorting (FACS) tubes, and finally analyzed with a FACScan flow cytometer (Becton, Dickinson and Company, USA).

**RESULTS**

**Construction of expression plasmid of human PDGF-B pronucleus**

In this study, we successfully isolated PDGF-B cDNA from human placenta by RT-PCR. The sequence of the amplified PDGF-B was confirmed by DNA sequencing, which was identical with the human PDGF-B described in the Genebank (NM_002608). The RT-PCR product and a part of sequencing map of PDGF-B are shown in Fig. 2. Mature fragments were cloned into the expression vectors, constructing the PDGF-B expression plasmid pET-PDGF-B under the control of T7 promoter.

**Expression, purification and refolding of pET-PDGF-B**

One major aim of this study was to produce recombinant PDGF-B proteins in prokaryote E. coli bacteria. We transformed the expression vector pET28a containing fragments of PDGF-B cDNAs into E. coli bacteria (strain BL21). Recombinant PDGF-B proteins were expressed at levels about 21.5% of total bacteria proteins by gel scanning. SDS-PAGE analysis revealed that recombinant proteins displayed a molecular weight of about 27 kDa (Fig. 3), which in general agreed with the deduced values. These results indicated that the recombinant proteins were correctly synthesized. Ni²⁺-NTA was sufficient to purify the recombinant proteins. In the refolding process, we found that the standard dialysis and dilution refolding procedures after inclusion body solubilization had many defects, such as long-time requirements, too many steps, inability to remove denaturing agents, and low refolding efficiency. Therefore, we used a system that allowed a faster purification and, ideally, that would facilitate the renaturation. A single-step refolding method was designed on Ni²⁺-NTA column with Sepharose NTA-Ni²⁺ superflow as the chromatographic matrix. An oligo-histidine domain fused to the N- or C-terminus of a recombinant protein overexpressed in E. coli has been reported to facilitate the purification of the studied protein by metalchelate chromatography. The purification was based on the affinity interaction between the oligo-histidine domain and the free ligand binding sites of an immobilized transition metal ion. This affinity interaction was also reported to be very strong under a variety of chemical conditions, allowing purification under denaturing conditions and development of a matrix-assisted renaturation. The advanced refolding method took less time, fewer steps and produced higher quantities of fully-renatured proteins than standard dialysis and dilution renaturation procedures. After purification and refolding on Ni²⁺-NTA column, PDGF-B had a purity of about 90%. But this advanced method relied on a presumption that the properly-folded form of a protein was thermodynamically or at least kinetically more stable compared with its unfolded or misfolded forms.

**Immunocytochemical staining of cat corneal endothelium**

In order to identify the primarily cultured cells being corneal endothelial cells, immunocytochemical staining of NSE was performed. NSE is the specific marker protein of cat corneal endothelium, which can effectively distinguish endothelial cells and keratocytes.
Corneal endothelial cells could keep expressing NSE even after passaging for 20 times, while keratocytes never expressed NSE. With NSE staining,uffy macrobeads could be found in the cytoplasm of corneal endothelial cells with over 98% positive rate, while NSE staining was negative in keratocytes in the control group (Fig. 4).

**Biological activities of expressed PDGF-B**

The other major aim of this study was to examine the bioactivity of the expressed products in the transfected cat endothelial cells. The effects of recombinant PDGF-B in promoting the survival and proliferation of cat endothelial cells were tested by MTT and flow cytometric assay. The MTT results showed that 75 ng/ml recombinant PDGF-B could obviously promote the survival of cat endothelial cells 7 days after culture ($P<0.05$ as compared with the controls), and the effects of 100 ng/ml PDGF-B on endothelial cells were more significant ($P<0.01$ as compared with the controls, Figs. 5 and 6). Cell proliferation was tested by flow cytometry. Corneal endothelial cells were treated with 100 ng/ml recombinant PDGF-B and DMEM for 7 days in the experimental groups, while in the control group no PDGF-B was used. The results of flow cytometry showed that PDGF-B could significantly enhance the proliferation of corneal endothelial cells, and G2/M phase cells were increased from 10.82% in the control group to 47.05% in the PDGF-B group ($P<0.001$, Fig. 7).

**DISCUSSION**

Corneal endothelium is essential for the maintenance of normal corneal hydration, thickness, and transparency.\(^\text{11,12}\) Despite the critical importance of this cell layer, a number of studies have shown that human corneal endothelial cells do not divide at a rate sufficient to maintain a constant density and that endothelial cell density tends to decrease with the increasing ages and after ocular insults.\(^\text{13,14}\) A disorder in the corneal endothelium caused by diseases or traumas can result in hydration of the stroma components and thus induce the onset of edema. In response to an injury, the endothelial cells may migrate into the wound site to establish partial tight junctions before the reestablishment of pump function and the restoration of corneal clarity. In human beings, corneal endothelium appears to heal primarily by enlargement and migration, not by proliferation of the endothelial cell layer.\(^\text{14-16}\) However, decompensation appears when the density of the corneal endothelial cells is lower than 25%-45% of the normal ones.

Although corneal transplantation is an effective treatment method for dysfunctional corneal endothelium, a few problems still remain with this approach. The primary one is the limited donor corneas for transplantation. Endothelial cell replacement may eliminate some of these problems by using tissue cultured cells as the donor source. Although many attempts have been made to establish tissue-cultured models in recent years, the establishment of pure and long-term cultured human corneal endothelial cells from adult donors has remained a challenging problem. Existing culture methods require young donors\(^\text{17,18}\) or special selection procedures to remove the contaminated fibroblasts.\(^\text{19}\)

In this study, we established monolayer cat corneal endothelial cells in relatively shorter time and higher cell density through applying recombinant PDGF-B to promoting the mitogenic activity. At the same time, method of tearing the decemets’ membrane established the primary cultures of cat corneal endothelial cells, which were free of contaminating fibroblasts. Cats were used because they mimicked more closely as the human beings in having little or no mitotic activity and a limited regenerative capacity compared with the highly-proliferative bovine and rabbit corneal endothelial cells.\(^\text{20}\)

In a word, we successfully constructed prokaryotic expression vector pET-PDGF-B and purified and renatured the recombinant PDGF-B protein and made it express in E. coli bacteria (strain BL21), then applied PDGF-B to promote the proliferation and differentiation of cat
corneal endothelial cells in vitro. The results of our study provide a foundation for biologically producing PDGF-B protein and further studying its function in reconstruction and trauma repair of human corneal endothelial cells.

Fig. 4. A: NSE staining is negative in cat keratocytes (immunocytochemical staining×400). B: NSE staining is positive in cat endothelial cells (immunocytochemical staining×400).

Fig. 5. A: Cat endothelial cells cultured in DMEM control media for 7 days. B: Cat endothelial cells cultured in DMEM plus 100 ng/ml recombinant PDGF-B for 7 days (phase contrast microscope×200).

Fig. 6. Effects of different concentrations of PDGF-B on survival of cat endothelial cells.

Fig. 7. Flow cytometric analysis of proliferation of corneal endothelial cells. PDGF-B significantly enhances proliferation of corneal endothelial cells. G2/M phase cells are improved from 10.82% in the control group to 47.05% in PDGF-B treatment group (P<0.001).

REFERENCES


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