Introduction

Intervertebral disc degeneration (IDD) is a major cause of lower back pain\(^1\), which includes a serious compression of the nerve roots or the spinal cord. At present, surgical removal of the intervertebral disc is the most common and effective clinical treatment of IDD. Although surgery can immediately alleviate the suffering, it is risky and expensive\(^2\). Moreover, it cannot fundamentally target the causes of disc protrusion and lower back pain. Therefore, the prevention of IDD is the key to solving this problem.

The causes of IDD include loss of proteoglycan, water, and type II collagen\(^3\), genetic factors, age, and insufficient transport of metabolites\(^4\). Till date, molecular biotherapy is increasingly gaining attention as a potential therapy for IDD treatment. This strategy aims to prevent or reverse the degeneration of the extracellular matrix of the intervertebral disc. This would ultimately control the development of IDD\(^5\).

Platelet-derived growth factor (PDGF) is an active polypeptide in mammals that plays a key role in chondrogenesis and regulation of cartilage homeostasis. PDGF may induce a platelet-rich environment. It is constructed by two polypeptide chains (homo- or heterodimer) connected by disulfide bonds. Accordingly, PDGF-B is one of the homodimeric structures. There have been several studies focus on the effects of PDGF-B on IDD. Thompson et al.\(^6\) performed in vitro experiments with mature dog intervertebral disc cells and demonstrated that mitosis and proteoglycan synthesis can be regulated by PDGF-B. In addition, it acts as an inhibitor of apoptosis\(^7\), which may protect the intervertebral disc cells from death. Rabbit experiments revealed PDGF-B treatment is able to maintain the intervertebral disc (IVD) architecture and limit the disruption of the boundary between the nucleus pulposus and the annulus fibrosus, similar to other growth factors\(^8\). As early as 2009, PDGF-B has been applied for orthopedic therapies\(^9\). However, whether PDGF, as a mitotic factor, could affect or prevent IDD through the nucleus pulposus cells (NPCs) has not been fully revealed. Till date, there has been no detailed report on the impact of PDGF-B on the function of the NPCs. This information would greatly assist in understanding the role of PDGF-B in IDD.

The present study investigated the in vitro effects of endogenous PDGF-B overexpression on the NPCs, including proliferation, matrix synthesis, and expression of the chondrogenic-function associated genes.

Materials and Methods

Isolation and culture of rat nucleus pulposus cells

The NPCs were obtained from four-week-old Sprague Dawley (SD) rats. Each rat was sacrificed followed by sterilization with 75% ethanol. The tail was removed and washed with phosphate buffered saline (PBS). The intervertebral disc annulus was opened, and the jelly-like nucleus tissue was scraped out with a sterile spoon. The tissue was soaked in a 6-cm dish. The medium was replaced every 3 days. All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (No.2016-108, 2016.12.06) and were carried out in strict accordance with Declaration of Helsinki (1964) and Laboratory Animal – Guidelines for ethical review of animal wel-
PDGF-B overexpression and analysis of cell transfection by fluorescence microscopy

According to the sequence information from China National GeneBank, the target gene was asked to be synthesized and then packaged into the lentiviral plasmid pLV-EGFP(2A) Puro by the Cyagen Bioscience. 293FT cells in a logarithmic growth phase were used for transfection. The lentiviral packaging helper plasmid pLP1, pLP2, and pLP-VSVG mixture was co-transfected with the constructed pLV-PDGFB plasmid into the 293FT cells using the Lipofectamine 2000 transfection reagent. The culture supernatant was collected after 72 h, centrifuged at 3,000 rpm for 15 min, and then filtered through a 0.45 μm filter to remove the cell debris. The purified virus was stored in a refrigerator at -80°C.

The rat NPCs were trypsinized and cell density was assessed. Then, the cells were seeded into a 6-well plate at a density of 2×10^5 cells per well and allowed to incubate overnight at 37°C, 5% CO_2. On the next day, the cells were washed twice with PBS and added to the complete medium containing the different viral mixtures. Approximately 48 h after transfection, the medium was removed, and fresh media containing 2.5 μg/ml puromycin was added to screen for the stably transfected cells. Negative control group was transfected with empty vectors. PDGF-B transfection efficiency was evaluated under an inverted fluorescence microscope. The medium was replaced every 3 days.

Real-time PCR

Total RNA was isolated from two groups of rat NPCs using Trizol reagent, and the concentration of each RNA sample was determined using a NanoVue Plus spectrophotometer (GE Healthcare Bio-Sciences AB, Sweden). All RNA samples were subsequently adjusted to the same concentration. A SYBR PrimeScript RT-PCR Kit (TaKaRa, Dalian, China) was then used for reverse transcription-PCR (RT-PCR) in accordance with the manufacturer’s protocol. Relative mRNA expression of PDGF-B was analyzed by real-time PCR using the IQ™5 System (Bio-Rad, USA) with β-actin serving as the reference gene. The relative expression of PDGF-B was calculated using the “normalized relative quantification” method followed by the 2^ΔΔct cycle threshold method. PCR reactions were performed in triplicate for each sample.

Western blot

For western blot, cells at 90% confluence were harvested and washed in PBS before incubation with RIPA lysis buffer on ice for 10 min. The whole cell lysates were clarified by centrifugation at 9,000 rpm for 10 min, and the supernatants were collected. The protein concentration was measured by BCA assay (Aidlab, Beijing, China). Equal amounts of total protein were separated by electrophoresis on 10% SDS-PAGE gel and then transferred to nitrocellulose membranes. They were subsequently blocked with TBST buffer containing 5% nonfat milk or 5% BSA for 2 h at room temperature and then incubated with primary antibodies for 3 h: anti-PDGFB-B (ab178409; Abcam), anti-β-actin (ab73630; Abcam), anti-Collagen-II (ABIN2506731, Biotin), and anti-Agrecan (100693-T08; Sino Biological), and anti-Collagen-X (LS-C208498, LSBio). Then the nitrocellulose membrane was washed three times with TBST buffer and then subjected to secondary Antibodies (HRP-conjugated goat anti-rabbit, 1:2,0000) for 1 h at room temperature. The immunoreactive proteins were visualized using the ECL detection system. The western blot experiments were repeated thrice.

Cell viability analysis

Cell viability was evaluated using the WST tetrazolium salt method (CCK-8, Dojindo) in accordance with the manufacturer’s instructions. The rat NPCs were seeded at 5,000 cells/well in the 96-well plates (Thermo Fisher, USA). PDGF-B were transfected into NPCs in experimental group and there was no intervention for negative control group. They were incubated for 2, 4 and 7 days respectively. Subsequently, 100 μl of the 10% CCK-8 solution was added to each well. These were then incubated for 4 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, USA). The cell viability was calculated using the OD values. All the experiments were performed in triplicate.

Collagen II Immunohistochemistry

NPCs were embedded in the 6-well plate at 5,000 cells/well for 4 days and 7 days. PDGF-B was transfected into the NPCs, while there was no intervention for negative control group. On the harvest day, medium was extracted and the cells were washed with PBS for 2 times, each time for 3 min. After being fixed with 4% paraformaldehyde for 20 min on ice, the cells were washed by PBS solution again for 2 times, each time for 3 min. The cells were then incubated with 5% TritonX-100 for 20 min and washed with PBS, following the protocol above. The cells were then blocked with fetal bovine serum for 1 h and washed, following the protocol above. The cells were added with primary antibody, anti-Collagen-II (ABIN2506731, Biotin), and incubated at 4°C overnight. The cells were washed with PBS solution for three times, each time for 10 min. Then added the secondary antibody solution and incubate the cells at room temperature for 1 h. Then the cells was incubated with horseradish enzyme labeled liquid at room temperature for 30 min. DAB chromogenic in dark enviroment for 5 min, until brown tissue observed under the microscope. The cells was finally washed, dehydrated, transparented and sealed. Collagen II expression was evaluated under an inverted phase contrast microscope.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software package (SPSS Inc., USA). The numeric data were expressed as the mean ± standard deviation (SD). The differences between the groups were analyzed using Student’s t-test or one-way analysis of variance. P values <0.05 were considered statistically significant.

Results

qRT-PCR and western blot analysis of PDGF-B expression

As shown in Fig. 1, mRNA expression of PDGF-B was significantly higher in the PDGF-B overexpression group than in the control group (P<0.05). These results suggested that PDGF-B was successfully transfected into the NPCs and efficiently expressed. Meanwhile, western blot analysis showed that PDGF-B was significantly upregulated in the PDGF-B groups in comparison with the control (Fig. 2).

Analysis of cell transfection by fluorescence microscopy

EGFP was used as the indicator for assessing PDGF-B transfection efficiency under an inverted fluorescence microscope. After 48 h of transfection, the stronger fluorescence was observed in the PDGF-B overexpression group, but not in the control group (Fig. 3).

PDGF-B overexpression promoted cell proliferation

The CCK8 assay was performed to investigate whether PDGF-B has a positive effect on the proliferation of the NPCs. Fig. 4 shows that cell viability was significantly increased in the PDGF-B groups compared to
PDGF-B overexpression impacted the expression of chondrogenic proteins

Western blotting was performed to evaluate the expression levels of the chondrogenic genes including collagen II, aggrecan, and collagen X (Fig. 5). The relative expression levels were calculated in comparison with the control group on day 4. The expression levels of both type II collagen and aggrecan were upregulated by the overexpression of PDGF-B, while collagen X expression was downregulated.

Collagen II Immunohistochemistry

To further evaluate the chondrogenic activity, collagen II immunohistochemistry staining was performed on day 4 and 7 after transfection. On day 4, the collagen II staining was slightly enhanced in the PDGF-B overexpressed cells than in the control group. Additionally, on day 7, a significant increase in the number of collagen II-positive cells was observed in the PDGF-B group (Fig. 6).
Lentiviral vectors are widely used for cell transfection studies due to and metastasis of IDD, the lentiviral vector of PDGF-B was constructed. PDGF-B-related mechanisms underlying the occurrence, development, and expression of chondrogenic genes in the NPCs. In order to reveal the effects of endogenous PDGF-B on biological functions and expression, injection therapy perfectly, PDGF-B was used as an exogenous stimulator. The purpose of this study was to investigate the effects of endogenous PDGF-B on biological functions and expression of chondrogenic genes in the NPCs. In order to reveal the PDGF-B-related mechanisms underlying the occurrence, development, and metastasis of IDD, the lentiviral vector of PDGF-B was constructed. Lentiviral vectors are widely used for cell transfection studies due to their stable cell transfection ability. In the present study, we hypothesized that PDGF-B can efficiently function in an intracellular environment, which might reveal a potential application in gene therapy. The protocol of the study suggested that the pLP-PDGF-B lentiviral vector could be successfully transfected into the rat IDD. Finally, PDGF-B was expressed in a stable manner.

On one hand, CCK8 results indicated that PDGF-B promoted the proliferation of the NPCs in a time-dependent manner. On the other hand, PDGF-B overexpression was able to synthesize the components according to the corresponding distribution. The intervertebral disc tissue includes the annulus fibrosus and nucleus pulposus. The annulus fibrosus is a framework of fibers arranged in concentric circles, and its structure exhibits type I collagen to type II collagen from the outside gradually to the inside. The nucleus pulposus at the center of the concentric structure is a loose network composed of mainly type II collagen and elastin fibers. It is rich in proteoglycans, especially aggrecan. Based on this, a key point of cytokine therapy is to promote its organization via the corresponding components according to their distribution. One of the major causes of IDD development is the loss of type II collagen. It has been vividly demonstrated in the western blot analysis, that the expression of both type II collagen and aggrecan is upregulated by the overexpression of PDGF-B. This exactly matches the components of the nucleus pulposus. Further investigation of the hypothesis was conducted using immunohistochemical staining to compare the amount of type II collagen between two groups. It was observed that on day 7, the type II collagen was significantly stronger under PDGF-B overexpression.

Numerous in vitro studies have shown that treatment with PDGF-B significantly inhibits cell apoptosis. Some studies indicated that PDGF promotes cell survival through the PI3 kinase-Akt signaling pathway. The resulting phosphorylation of NF-kB is the immediate cause for the inhibition of apoptosis. Montaseri et al. suggested the PDGF-B suppresses IL-1B-induced cartilage degradation by downregulation of NF-kB signaling. These reports concur with the results of the present study, which demonstrated the ability of PDGF-B to enhance cell proliferation and upregulate the expression of the chondrogenic proteins. Recently, an increasing number of researchers are beginning to pay attention to the positive effects of platelet-rich plasma (PRP) on the NPCs. The potential effect of PRP is the initiation of the process of angiogenesis, followed by bone regeneration with the increased blood supply. As an important constituent of PRP, the different roles played by PDGF in angiogenesis have been displayed in various applications. The micro-vascularization of IVD enhanced by PDGF might also contribute to the result.

This study shows the important role of PDGF-B in enhancing NPC proliferation. However, it still has several limitations. The culture was propagated for approximately one week in the present study. Thus, the long-term effect of PDGF-B on the NPCs is still unknown. Besides, the specific biochemical pathways in which PDGF-B plays its role were not determined. Despite these limitations, the results of this study offer evidence regarding the potential application of PDGF-B in gene therapy. Thus, future studies focusing on the precise mechanism and pathway of PDGF-B can be undertaken.

In summary, PDGF-B overexpression through lentiviral transfection could promote the proliferation of NPCs and enhance matrix synthesis. Thus, PDGF-B could be a promising target to delay or reverse IDD.

Discussion

Previous studies have demonstrated the proliferative effects of PDGF-B on IVD cells in vitro. Although these studies simulated the injection therapy perfectly, PDGF-B was used as an exogenous stimulus. In the present study, we hypothesized that PDGF-B can efficiently function in an intracellular environment, which might reveal a potential application in gene therapy. The purpose of this study was to investigate the effects of endogenous PDGF-B on biological functions and expression of chondrogenic genes in the NPCs. In order to reveal the PDGF-B-related mechanisms underlying the occurrence, development, and metastasis of IDD, the lentiviral vector of PDGF-B was constructed. Lentiviral vectors are widely used for cell transfection studies due to their stable cell transfection ability. Here, the most commonly used method in lentivirus packaging was followed. This included PCR amplification of the target gene, identification, vector ligation, viral vector generation by transient transfection of 293T cells, virus transfection, and screening of the stably transfected cells (target highly expressed). The protocol of the study suggested that the pLP-PDGF-B lentiviral vector could be successfully transfected into the rat IDD. Finally, PDGF-B was expressed in a stable manner.

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It can be suggested that PDGF-B could be of assistance in therapy since it can replenish the lost components of degenerative NPC.

Type X collagen is a biochemical component of the IVD matrix and is mainly expressed in the cartilaginous endplate. It has been widely reported the type X collagen is associated with osteoarthritis, and usually causes the expression of the hypertrophic chondrocyte phenotype. The expression and deposition of type X collagen are mainly related to aging. Similarly, with aging in scoliosis, some cells from the nucleus pulposus differentiate to the hypertrophic chondrocyte phenotype, accompanied by the overexpression of collagen X. According to Itoh et al., enhanced expression of type X collagen could be found in the extruded nucleus pulposus of the chondrodystrophoid dog. Type X collagen can be deemed as an indicator of NPC aging and pathological changes. As revealed in the western blot results, the expression of type X collagen was suppressed, strongly indicating a decrease in NPC degeneration. Therefore, PDGF-B could be beneficial for the NPCs as it delays and even reverses their degeneration via the chondrogenic pathways.

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Conflict of Interest

The authors have declared that no COI exists.

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