Original

Effects of PDGF-B Overexpression on the Biological Activity of Nucleus Pulposus Cells

Jun Ge*, Minfeng Gan*, Cenhao Wu, Qi Yan, Yufeng Chen, Huilin Yang and Jun Zou

Department of Orthopaedic Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China

(Received for publication, February 2, 2019)

Abstract: The development of intervertebral disc degeneration (IDD) is largely influenced by disorders in the nucleus pulposus cells (NPCs) and collagen loss. This study aimed to investigate the in vitro effects of the overexpression of the platelet-derived growth factor (PDGF)-B on the biological functions and chondrogenic gene expression in the NPCs. PDGF-B was overexpressed in primary cultured rat NPCs using lentivirus. The CCK8 method was used to assess in vitro cell viability that indicates the proliferation activity. Real-time polymerase chain reaction (PCR) and western blotting were applied to observe the matrix synthesis and expression of the chondrogenic genes. Cell viability was significantly increased in the PDGF-B groups compared to the negative controls in a time- and dose-dependent manner. PDGF-B enhanced mRNA expression of collagen II and aggrecan, and suppressed that of collagen X. On day 7, the number of collagen II-positive cells significantly increased in the PDGF-B group. Taken together, PDGF-B overexpression could promote the proliferation of NPCs and enhance matrix synthesis in vitro. Thus, PDGF-B can be a promising target to prevent and reverse IDD.

Key words: Intervertebral disc degeneration, Matrix synthesis, Nucleus pulposus cell, Platelet-derived growth factor-B.

Introduction

Intervertebral disc degeneration (IDD) is a major cause of lower back pain1, 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 expensive1. 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 collagen3, genetic factors, age, and insufficient transport of metabolites3. 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 IDD3.

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.8 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 apoptosis9, 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 factors6,7. As early as 2009, PDGF-B has been applied for orthopedic therapies3. 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 and cultured in DMEM/F12 (1:1) supplemented with 15% FBS at 37ºC for 2 h, during which it was gently shaken. Subsequently, the tissue was digested with an amount of 0.2% type II collagenase. The mixture was digested at 37ºC for 2 h, during which it was gently shaken. Subsequently, the serum-containing medium was added to terminate the digestion. The solution was centrifuged at 1,000 rpm for 5 min, and the cells were washed twice with DMEM/F12 (1:1). Thereafter, the cells were resuspended and cultured in DMEM/F12 (1:1) supplemented with 15% FBS 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 2×10^4 cells per well and allowed to incubate overnight at 37°C, 5% CO₂. 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. 2.5 ug/ml puromycin was added to screen for the stably transfected cells. Approximately 48 h after transfection, 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.

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

As shown in Fig. 1, 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.

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
Jun Ge et al.: PDGF-B Overexpression and Nucleus Pulposus Cells

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).
their stable cell transfection ability. Lentiviral vectors are widely used for cell transfection studies due to their transduction ability and metastasis of IDD, the lentiviral vector of PDGF-B was constructed. PDGF-B-related mechanisms underlying the occurrence, development, and pathogenesis of chondrogenic genes in the NPCs. In order to reveal the effects of endogenous PDGF-B on biological functions and expression of chondrogenic genes in the NPCs, at
day 7, a significant increase in the number of collagen II-positive cells was observed in the PDGF-B group.

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.

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. 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.

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 in vitro. 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 in vitro. Thus, PDGF-B could be a promising target to delay or reverse IDD.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81472132, 81572183 and 81672220), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflict of Interest

The authors have declared that no COI exists.

Reference

1. Frymoyer JW and Cats-Baril WL. An overview of the incidences
and costs of low back pain. Orthop Clin N Am 22: 263-271, 1991
2. Yoon ST and Patel NM. Molecular therapy of the intervertebral disc. Eur Spine J 15 Suppl 3: S379, 2006
3. Adams MA and Roughley PJ. What is intervertebral disc degeneration, and what causes it? Spine 31: 2151-2161, 2006
4. Thompson JP, Jr OT and Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. Spine 16: 253-260, 1991
5. Gruber HE, Norton HJ and Jr HE. Anti-apoptotic effects of IGF-1 and PDGF on human intervertebral disc cells in vitro. Spine 25: 2153-2157, 2000
6. Paglia DN, Singh H, Karukonda T, Drissi H and Moss IL. PDGF-BB Delays degeneration of the intervertebral discs in a rabbit pre-clinical model. Spine 41: E449, 2016
7. Leckie SK, Bechara BP and Hartman RA. Injection of AAV2-BMP2 and AAV2-TIMP1 into the nucleus pulposus slows the course of intervertebral disc degeneration in an in vivo rabbit model. Spine J 12: 7-20, 2012
8. Alzube L, Breithart EA, O’Connor JP, Parsons JR, Bradica G, Hart CE and Lin SS. Recombinant human platelet-derived growth factor BB (rhPDGF-BB) and beta-tricalcium phosphate/collagen matrix enhance fracture healing in a diabetic rat model. J Orthop Res 27: 1074-1081, 2010
9. Presciutti SM, Paglia DN, Karukonda T, Soung DY, Guzzo R, Drissi H and Moss IL. PDGF-BB inhibits intervertebral disc cell apoptosis in vitro. J Orthop Res 32: 1181-1188, 2014
10. Naldini L and Verma IM. Lentiviral vectors. Adv Virus Res 55: 599-609, 2000
11. Brickleyparsons D and Glimcher MJ. Is the chemistry of collagen in intervertebral discs an expression of Wolff’s Law? A study of the human lumbar spine. Spine 9: 148-163, 1984
12. Lammi P, Inkinen R, Der Mark KV, Puustjarvi K, Arokoski JPA, Hyttinen MM and Lammi MJ. Localization of type X collagen in the intervertebral disc of mature beagle dogs. Matrix Biol 17: 449-453, 1998
13. Walker GD, Fischer M, Gannon J, Jr TR and Jr OT. Expression of type-X collagen in osteoarthritis. J Orthop Res 13: 4-12, 2010
14. Aigner T, Reichenberger E, Bertling W, Kirsch T, Stöß H and Mark KVD. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch B Cell Pathol Incl Mol Pathol 63: 205-211, 1993
15. Eerola I, Salminen H, Lammi P, Lammi M, Mark KVD, Vuorio E and Säämänen AM. Type X collagen, a natural component of mouse articular cartilage: Association with growth, aging, and osteoarthritis. Arthritis Rheumatol 41: 1287-1295, 2010
16. Aigner T, Greskötter KR, Fairbank JCT, Mark KVD and Urban JPG. Variation with age in the pattern of type X collagen expression in normal and scoliotic human intervertebral discs. Calcified Tissue Int 63: 263-268, 1998
17. Itoh H, Asou Y, Hara Y, Haro H, Shinomiya K and Tagawa M. Enhanced type X collagen expression in the extruded nucleus pulposus of the chondrodystrophic dog. J Vet Med Sci 70: 37-42, 2008
18. Shimamura H, Terada Y, Okado T, Tanaka H, Inoshita S and Sasaki S. The PI3-kinase-Akt pathway promotes mesangial cell survival and inhibits apoptosis in vitro via NF-kappa B and Bad. J Am Soc Nephrol 14: 1427-1434, 2003
19. Montaseri A, Busch F, Mobasher A, Buhrmann C, Aldinger C, Rad JS and Shakibaei M. IGF-1 and PDGF-bb suppress IL-1β-induced cartilage degradation through down-regulation of NF-κB signaling: involvement of Src/PI-3K/AKT pathway. Plos One 6: e28663, 2011
20. Akeda K, An HS, Pichika R, Attawia M, Thonar EJ, Lenz ME, Uchida A and Masuda K. Platelet-rich plasma (PRP) stimulates the extracellular matrix metabolism of porcine nucleus pulposus and anulus fibrosus cells cultured in alginate beads. Spine 31: 959, 2006
21. Kim HJ, Jin SY, Koh YG, Yeo JE, Kang KT, Kang YM, Chang BS and Lee CK. Anti-inflammatory effect of platelet-rich plasma on nucleus pulposus cells with response of TNF-α and IL-1. J Orthop Res 32: 551-556, 2014
22. Yao Q, Renault MA, Chapoul Y, Vandierdonck S, Belloc I, Jaspar-Vinassa B, Daniel-Lamazière JM, Laffargue M, Merched A and Desgranges C. Sonic hedgehog mediates a novel pathway of PDGF-BB-dependent vessel maturation. Blood 123: 2429-2437, 2014
23. Gianni-Barrera R, Burger M, Wolff T, Heberer M, Schaefer DJ, Gürke L, Mujagic E and Banfi A. Long-term safety and stability of angiogenesis induced by balanced single-vector co-expression of PDGF-BB and VEGF164 in skeletal muscle. Sci Rep-UK 6: 21546, 2016
