Original Article
Role of growth differentiation factor-5 and bone morphogenetic protein type II receptor in the development of lumbar intervertebral disc degeneration

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Abstract: The present study was designed to evaluate the role of growth differentiation factor-5 (GDF-5) and bone morphogenetic protein type II receptor (BMPR-II) in the development of lumbar intervertebral disc degeneration (IDD). A total of 24 patients with lumbar IDD (experiment group) and 6 patients with lumbar vertebral fracture (control group) were enrolled in the study. Tissue samples of IVD from the experiment group and control group were obtained during lumbar fusion operation, respectively. Fixation and decalcification of IVD tissue were performed, and then HE staining was carried out to observe the morphological changes of the lumbar IVD tissues. The expression of GDF-5 and BMPRII in human lumbar IVD was detected by immunohistochemical staining. HE staining results showed that non- and minimal degeneration was found in 11 cases (score range, 0-3), moderate degeneration in 12 cases (score range, 4-8), and severe degeneration in 7 cases (score range, 9-12). According to the immunohistochemical results, the positive expression rates of GDF-5 and BMPRII in NP were higher than those in AF of the non- and minimal degeneration group, moderate degeneration group and severe degeneration group (all $P < 0.05$). However, no significant difference in GDF-5 or BMPRII positive expression was observed among the normal, non- and minimal, moderate and severe degeneration groups in neither NP area nor AF area (all $P > 0.05$). In conclusion, our results showed that GDF-5 and BMPRII expressed both in normal and degenerated IVD tissues, and GDF-5 might have an inhibition effect on degenerated lumbar IVD, suggesting that gene therapy may be a useful approach in producing physiological effects during early- and late-phase of lumbar IDD.

Keywords: Growth differentiation factor-5, bone morphogenetic protein type II receptor, lumbar intervertebral disc degeneration, intervertebral disc

Introduction

Degenerative disc disease (DDD), a kind of chronic low back pain syndrome caused by intervertebral disc degeneration (IDD), had several categories, including discogenic low back pain, lumbar instability, lumbar spinal stenosis and lumbar disc herniation (LDH) [1-3]. As the commonest DDD, LDH had an incidence rate of 7.62% every year in the world, and people aged between 25 and 55 are associated with higher risk of LDH, resulting in great social, healthcare and economic burden [4, 5]. Meanwhile, DDD, as one of the major causes of chronic lower back pain, is characterized by a number of pathophysiological features, including lumbago, sciatica, cauda equina symptoms, as well as the senescence, apoptosis and endplate calcification of nucleus and endplate cartilage cells, and the decline of the extracellular matrix (ECM) like type II collagen and proteoglycan, which may play important roles in maintaining disc functions [6-8]. Certainly, there are various risk factors of DDD, such as lumbar disc degeneration, injury, the anatomical factors on intervertebral disc's own weakness, genetic factors, lumbosacral congenital anomalies [9, 10]. Additionally, previous studies have shown that a variety of cytokines play an important role in the inhibition of DDD process, such as GDF-5, TGF-β, EGF, BMPs, IGF, FGF, etc. [11, 12].

Growth differentiation factor 5 (GDF-5), a member of the bone morphogenetic protein (BMP)

family, is also referred to cartilage-derived morphogenetic protein-1 or BMP-14 [13, 14]. GDF-5 is a preprotein synthesis composed of 501 amino acids in human, and plays a regulatory role in promoting proliferations of osteoblast, periosteum cells, and connective tissue fibroblasts [15, 16]. Furthermore, GDF-5 has been extensively reported to enhance endochondral bone growth, normal development of limb skeletons and joints, and odontogenesis [17, 18]. It has also been confirmed previously that GDF-5 enables regeneration and suppresses degeneration by intensify production of viable cells and matrix synthesis [19]. The underlying mechanisms may lie in that GDF-5 can stimulate proteoglycans (PG) and type II collagen production in intervertebral disc (IVD) cells, and enhance cell proliferation and matrix synthesis in annulus fibrosus (AF) and nucleus pulposus (NP) cells, so as to inhibit IDD correspondingly [16, 20]. GDF-5 also binds to BMP type II receptor (BMPR-II) and thought to play an important role in bone morphogenesis [13]. Bone morphogenetic protein-2 (BMP-2) is known to be glycosylated polypeptide with 396 amino acids, and has greater osteoinductive activity than other BMPs [15]. Previous studies have showed that BMP-2 may be associated with orthotopic or ectopic bone formation and the osteogenic differentiation of mesenchymal stem cells [17, 21]. Regenerative activity of BMP-2 has been unraveled by sufficient studies in animal models, including such aspects as bone formation, connective tissue attachment and cementum formation [18, 22, 23]. In addition, absence of BMP-2 has been demonstrated in blood vessels of IVD, but BMP-2 has been found in NP cells of degenerated IVD, indicating that BMP-2 may be not involved in degeneration but in regeneration of IVD [24, 25]. It has also been suggested that BMP-2 can promote PG synthesis and ECM production in the articular chondrocytes and IVD [26]. In the current study, we intended to investigate the expression of GDF-5 and BMP-2 in degenerated IVD, and to analyze the mutual correlation of GDF-5 and BMP-2 expressions with the degree of IDD, providing optimize treatment strategies and propose new gene therapies for DDD.

Materials and methods

Ethics statement

The study was performed after the Institutional Review Board of Tongren Hospital Affiliated to Jiaotong University School of Medicine gave written permission. The informed written consent was obtained from each eligible participant and all procedures were conducted according to the Declaration of Helsinki.

Study subjects

From March 2012 to May 2013, a total of 24 patients with lumbar IDD (experiment group) were randomly recruited from the Department of Orthopedic Surgery, Tongren Hospital Affiliated to Jiaotong University School of Medicine; the enrolled patients should meet all the following criteria: (1) the diagnosis of lumbar IDD were confirmed by imaging examination; (2) had failed conservative treatment for lumbar IDD; (3) patients were in grades II~V according to the Pfirrmann Grading System for Lumbar Disc Degeneration [27]. Among the 24 included patients, there were 7 patients with lumbar disc protrusion, 7 patients with lumbar spinal stenosis and 10 patients with lumbar instability and lumbar spondylolisthesis. In addition, 6 young patients with lumbar vertebral fracture (Pfirrmann grade I) were enrolled as the control group if they showed evidence of proper surgical indications for lumbar fusion operation. Subjects with hypertension, diabetes mellitus and related microvascular diseases were excluded.

Fixation and decalcification of IVD tissue

Tissue samples of IVD from the experiment group and control group were obtained during lumbar fusion operation, respectively. Tissue samples were fixed in 10% neutral formalin for 24~48 hr, placed in ampoule containing with 10% EDTA, blocked with cap, and then immersed in a water bath; decalcification of tissues was conducted with the assistance of microwave oven at 37~42°C for 2 h, and the original solution was replaced by 15% EDTA; further incubation were carried out in a thermostatic water bath at 37°C overnight; 15% EDTA were changed again on the following day, and further decalcification was performed using microwave oven; decalcification of IVD tissue was completed after 15~20 day, and for endplate cartilage tissue, decalcification was finished after 20~35 day.

HE staining

After fixation and decalcification, paraffin-embedded lumbar IVD tissues were cut into 4
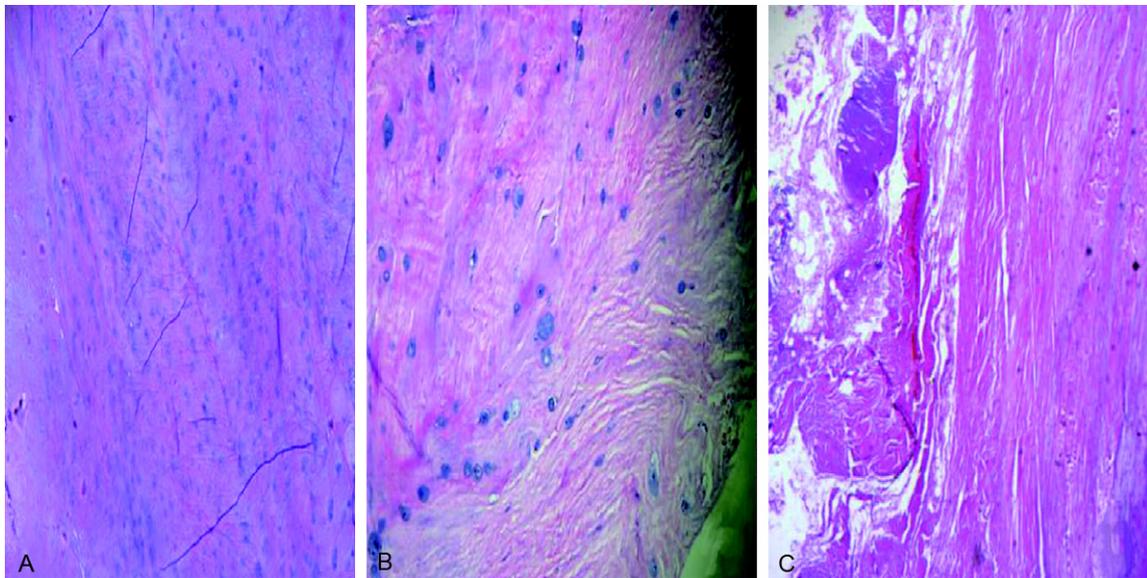
Figure 1. H&E staining and histological grade of lumbar IVD tissue.
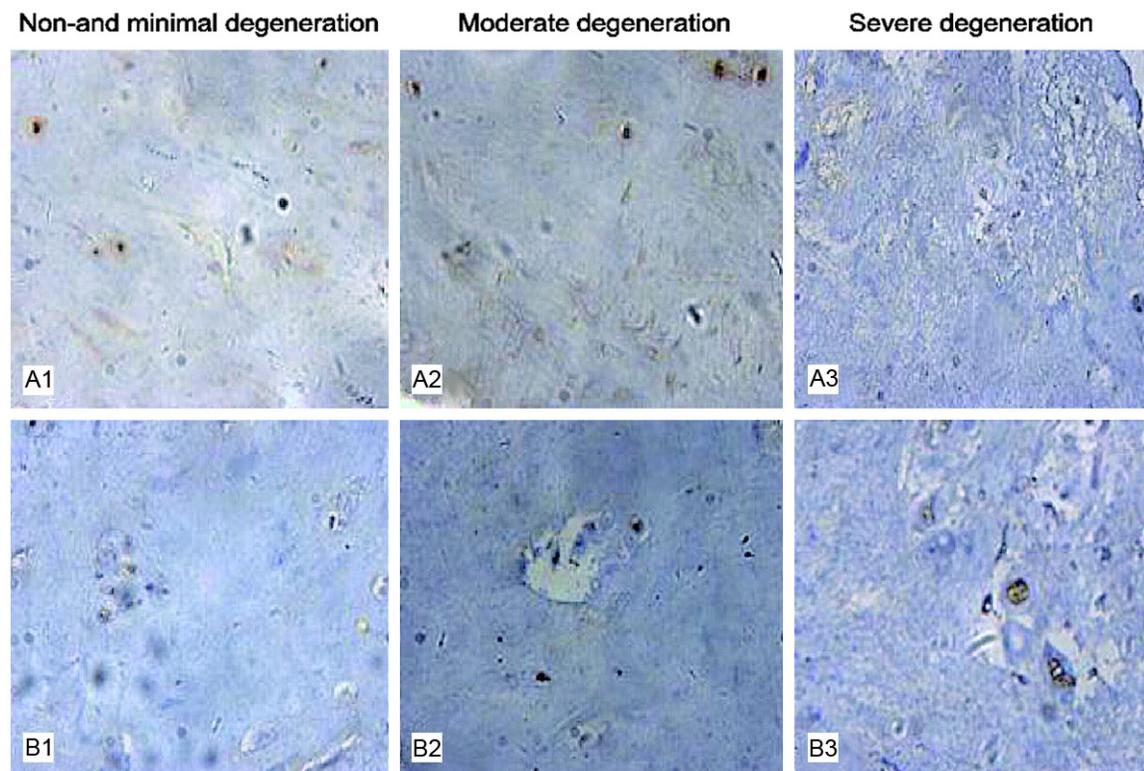
Figure 2. Immunohistochemical staining for GDF-5 and BMPRII in human lumbar IVD; brown indicates a positive reaction; GDF-5 (row A), BMPRII (row B).

μm slices; the sections were deparaffinized in xylene solution, stained by using hematoxylin and eosin (HE) and sealed with neutral gum. Coronal slices of each IVD tissue were observed under the microscope. Coronal slices were stained with nuclear blue and cytoplasm light red. The slices were scored for the degree of degeneration on the basis of histological appearances, the scoring system was: no/minimal degeneration (score 0~3); moderate

Table 1. Positive rate of GDF-5 and BMPRII expression in lumbar IVD tissue

	Non- and minimal degeneration	Moderate degeneration	Severe degeneration
GDF-5 (NP)	62.09 ± 10.04**,##	55.0 ± 9.16##	56.71 ± 2.43
GDF-5 (AF)	33.27 ± 7.36*,**,##	30.08 ± 6.16*,##	28.14 ± 5.87*
BMPRII (NP)	34.64 ± 5.95**,##	38.75 ± 7.02##	36.43 ± 3.37
BMPRII (AF)	26.18 ± 8.36#,**,##,	29.67 ± 6.41#,##	21.57 ± 4.24#

NP, nucleus pulposus; AF, annulus fibrosus; *, compared with GDF-5 (NP); $P < 0.05$; **, compared with moderate degeneration group; $P > 0.05$; #, compared with BMPRII (NP); $P < 0.05$; ##, compared with severe degeneration group; $P > 0.05$.

degeneration (score 0~8); severe degeneration (score 9~12) [28].

Immunohistochemistry

After being placed in baking box at 60°C for 10 min, the tissue sections were daparaffinized in xylene and washed three times with PBS. Slices were immersed in the citrate antigen retrieval solution and placed in a microwave oven for 13 min at 500 W for antigen retrieval. After rinsed three times with PBS, slices were supplemented with H_2O_2 solution for eliminating the endogenous peroxidase activity, and then incubated in 50 μL endogenous antagonist (solution A) for 15 min. After the removal of solution A, further incubation was carried out in 50 μL lowlenthal serum (solution B) for 10 min and then serum was discharged. Each slice was supplemented with 50 μL mouse anti-human GDF5/BMPR2 (20 μg/ml, Pepro Tech) as first antibody, and incubated at 37°C for 60 min in a water bath. Then, 50 μL biotin-labeled second antibody was added and incubated at room temperature for 10 min. After removing the PBS solution, 50 μL streptavidin-perosidase solution was added into the mixture and a forth incubation was performed at room temperature for 10 min. Subsequently, diaminobenzidine (DAB) color liquid was added. After 3~10 min, the slices were counterstained using hematoxylin. First antibody was replaced by PBS as the negative control. Protein expression of GDF-5 and BMPR II was observed under a light microscope. Slices of lumbar IVD tissues were divided in NP and AF. For NP and AF analysis, total 200 cells were collected from the highest density area of positive cells (dark brown granules in nucleus and cytoplasm), respectively. The percentage of staining positive cells in congener counted cells was calculated as the result of cells staining.

Statistical analysis

Statistical analysis was conducted by using the SPSS 18.0 software. Continuous variables with normal distribution were expressed as mean ± standard deviation (SD). Enumeration data was expressed by positive rate and comparisons between groups (no/minimal degeneration, moderate degeneration and severe degeneration; NP and AF) were applied one-way ANOVA. Results were considered statistically significant with $P < 0.05$.

Results

HE staining results

In order to observe the morphological changes of the lumbar IVD tissue, the HE staining was performed. **Figure 1** showed the results of HE staining. According to the histological performance, non- and minimal degeneration was found in 11 cases (score range, 0-3; average, 2), moderate degeneration in 12 cases (score range, 4-8; average, 6.83), and severe degeneration in 7 cases (score range, 9-12; average, 10.14). To be more specific, a clear boundary could be seen between NP and AF, no cracks or fissures existed in NP, and no cell cluster formation was found in the non- and minimal degeneration group. As for the 12 cases of moderate degeneration, the boundary between NP and AF became fuzzy, cracks extended to NP overlapping with AF, and 25-75% of the cells formed small clusters. In severe degeneration cases, the boundary between NP and AF disappeared, cracks extended to the lateral AF, and more than 75% cells formed clusters.

Expression of GDF-5 and BMPRII in lumbar IVD tissue

The positive expression signals of GDF-5 and BMPRII in lumbar IVD tissue were localized in the cytoplasm of cartilage cells, while there was no positive signal in the extracellular matrix (**Figure 2**). The positive expression rates of GDF-5 and BMPRII in NP were higher than those in AF in the non- and minimal degeneration group (GDF-5: 62.09 ± 10.04% vs. 33.27 ±

7.36%; BMPRII, 34.64 ± 5.95% vs. 26.18 ± 8.36%, respectively), moderate degeneration group (GDF-5: 55.0 ± 9.16% vs. 30.08 ± 6.16%; BMPRII, 38.75 ± 7.02% vs. 29.67 ± 6.41%, respectively) and severe degeneration group (GDF-5: 56.71 ± 2.43% vs. 28.14 ± 5.87%; BMPRII, 36.43 ± 3.37% vs. 21.57 ± 4.24%, respectively) (all P < 0.05). However, no significant difference in GDF-5 or BMPRII positive expression was observed among the normal, non- and minimal, moderate and severe degeneration groups in neither NP area nor AF area (all P > 0.05) (**Table 1**).

Discussion

In this study, we observed the expressions of both GDF-5 and BMPRII in IVD tissues and analyzed the correlation between these expressions and lumbar IDD, reporting that GDF-5 might have an inhibition effect on lumbar IDD. GDF-5 has been verified to be a suitable candidate having an impact on human IDD, and playing momentous control roles in chondrogenesis and joint formation [29-31]. Shen et al demonstrated the regulation and promotion ability of GDF-5 in both osteogenic and osteoblastic properties and thereby enhancing osteogenic differentiation [32]. In addition, according to the report of Zeng et al, GDF-5 could also increase the expression of vascular endothelial growth factor in vitro, consequently promoting the angiogenic activity in stroma cells [33]. A study indicated that increased rhGDF-5 was of the capability of repairing IVD, with the presumably mechanism of its up-regulation effect on ECM production in vitro [34]. Also, GDF-5 was manifested with the ability of up-regulating collagen type II as well as aggrecan and down-regulating MMP-3, which were parameters for IVD cell metabolism, to ultimately promote the proliferation of IVD cells and enhance the accumulation of ECM [35]. Therefore, we considered that GDF-5 could be regarded as a suitable actor for the gene therapy of lumbar IDD.

Our results indicated an apparent higher expression of GDF-5 and its receptor BMPRII in the central NP than those in edge AF. The calcification increase of cartilaginous endplate (CEP) results in reduced nutrient supply, which has been considered to be essential in the occurrence of IDD [36]. CEP, a thin hyaline cartilage layer between vertebral endplate and NP, has been illustrated to be a gateway to trans-

port nutrient from adjacent blood vessels into the discs [37]. The permeability of endplate could be increased with the loss of cartilage, contributing to endplate inflammation and subsequent disc infection [38]. The first stage of IDD is PG depletion, and the aggrecan loss from NP is capable of lowering resistance to compression, inducing decreased disc height and changed mechanical properties of IVD [39]. GDF-5 may only up-regulate the expression of type II collagen and aggrecan, also evoke the expansion of inner AF fibrochondrocyte populations into NP [40, 41]. That is, GDF-5 is able to stimulate the expression of PG and maintain the transport route of nutrients between endplates and IVD, thereby preventing the degeneration of endplates and IVD.

Results in our study also suggested that GDF-5 has been expressed both in normal and degenerated IDD tissues, yet the expression has nothing to do with the degree of degeneration. Based on the precedent evidence, GDF-5 cloning might be facilitative to our studies on the signaling pathways of GDF-5 in mouse IVD cells and might be helpful to explain the mechanism by which GDF-5 defect will lead to degenerative changes in the disc, besides, in light of reverse transcription PCR, we recognized that the relevant receptors like BMPR1A, BMPR1B, and BMPR2 were expressed in native human IVD tissue and in cultured IVD cells [16, 35, 42]. Although GDF-5 in normal, moderate and severe degeneration of IVD are expressed, the differences among different groups were not significant, which indicates that GDF-5 may play an crucial role in stabilizing the endogenous matrix in human IVD, and IDD was not caused by the reduction of intervertebral disc endogenous GDF-5 [34, 43, 44]. Furthermore, even though GDF-5 was widely presented in normal and degenerated human lumbar IVD, it was still unknown whether it has certain repairing effects or was possible to reverse IDD in the various periods of IDD in vivo on complex physiological and chemical conditions or not, hence, we could speculate that GDF-5 may be expressed both in normal and degenerated IVD in IDD patients, but the GDF-5 expression may not be relevant to the degree of degeneration [29, 41, 45].

In addition, as a receptor of GDF-5, expression of BMP-2 was found both in normal IVDs and degenerated IVDs, indicating that its expres-

sion level was uncorrelated with degeneration degrees. Collected data of our study reported that BMP-2, as a receptor of BMP family, presented significant higher expression in NP regions than AF regions, manifesting that BMPs and GDF-5 may influence human IVDs through NP cells [25, 46]. Nevertheless, the expression of BMP-2 in IVDs was not associated with degree of IDD under certain conditions, which suggested that all the growth factors in the BMP family, including GDF-5, were likely to exert physiological effects to NP cells in IVDs, either in the early stage of degeneration or the advanced stage. Specifically, BMP-2 enables favorable improvements in degenerated IVDs at the early stages and fails to improve the degeneration conditions at the advanced stage. H&E staining and immunohistochemistry findings showed that BMP-2 caused cartilage formation in some degenerated IVDs, whereas later findings didn't show improvement in IVDs [24, 40, 47, 48].

In conclusion, our results showed that GDF-5 might have an inhibition effect on degenerated human lumbar IVD, GDF-5 and BMPRII expressed both in normal and degenerated IVD tissues, suggesting that gene therapy may be a useful approach in producing physiological effects during early- and late-phase of lumbar IDD. However, further in vivo and in vitro studies are necessary for the identification of the potential roles of GDF-5 on lumbar IDD. And to further explore the molecular mechanisms of GDF-5 do contribute a lot to the deeper interpretation of the incidence and targeted treatment of lumbar IDD.

Acknowledgements

Disclosure of conflict of interest

None.

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