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Expression of glial cell line-derived neurotrophic factor in the human intervertebral disc

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Structured Abstract

Study Design. Biochemical and immunohistochemical analyses by the human intervertebral disc (IVD) cells and tissues.

Objective. To examine the expression of glial cell line-derived neurotrophic factor (GDNF) and its receptors, GDNF family receptor (GFR) α1 and rearranged during transfection (RET) in the human IVD cells and the tissues with the early and advanced stages of degeneration.

Summary of Background Data. The neurotrophin family, including nerve growth factor (NGF), has been reported to be expressed in the IVDs and plays a role in hyperalgesia and neuronal sensitization. Despite having properties similar to the NGF, the expression of GDNF in the IVD remains unknown.

Methods. Human IVD cells were cultured in monolayer. Immunohistochemical analyses and western blotting were performed to examine the protein levels of GDNF and its receptors. To examine the effect of proinflammatory cytokines, cells were cultured in the presence of interleukin-1β (IL-1β). The immunohistochemical expression of these proteins was also evaluated using human IVD tissues with different stages of degeneration.

Results. Immunofluorescent reactivity against anti-GDNF, GFRα1, and RET antibodies was identified in human IVD cells. In protein extracts from IVD cells, those protein expressions were also identified by Western blot. IL-1β significantly stimulated the mRNA expression of GDNF compared to that of the control group. There was no significant effect of IL-1β on the mRNA expression of GFRα1 and RET. The percentage of GDNF-immunopositive cells in advanced degenerated discs was significantly higher than that in early degenerated discs, whereas those of GFRα1 and RET showed no significant differences.

Conclusions. GDNF and its receptors were constitutively expressed in the human IVD cells. GDNF expression was significantly enhanced by proinflammatory stimuli, and in the microenvironment with advanced tissue degeneration.

Key Words: neurotrophin family; glial cell line-derived neurotrophic factor (GDNF); intervertebral disc; Low back pain

Level of Evidence: N/A

Key point

- The expression of glial cell line-derived neurotrophic factor (GDNF), GDNF family receptor (GFR) α1, and rearranged during transfection (RET) by the human intervertebral disc (IVD) was identified both at the mRNA and protein levels.
- mRNA expression of GDNF of human IVD cells was significantly upregulated by proinflammatory cytokine (IL-1β) stimulation.
- Percentage of GDNF immunopositive cells was significantly increased in the human IVD tissues with the advanced stage of degeneration compared to those with the early stage of degeneration.

INTRODUCTION

Intervertebral disc (IVD) degeneration is one of the critical factors responsible for low back pain [1]. Accumulating evidence has demonstrated that proinflammatory cytokines, such as interleukin-1 (IL-1) β and tumor necrosis factor-alpha (TNF-α), expressed by the degenerated IVD play a pivotal role in the process of tissue degradation by promoting chemokine production, changes in IVD cell phenotype and extracellular matrix degradation [2]. These cytokines, and the changes in the microenvironment of the IVD induced by inflammatory

cytokines, have been reported to be associated with the pathogenesis of discogenic low back pain by involving annular tears, migration of inflammatory cells and nerve ingrowth [2,3]. However, the exact mechanism of pain transmission remains unknown.

Neurotrophic factors (NFs) are a family of biomolecules that includes the neurotrophin family, glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), and neuropoietic cytokines [4]. These factors have a significant role in the growth and survival of both the peripheral and central nervous systems [4]. Importantly, NFs have also been shown to play a significant role in the transmission of physiologic and pathologic pain, including nociceptive and neuropathic pain [4,5].

Among the NFs, neurotrophins (nuerotrophin family) are known to take part in inflammatory responses and pain transmission by increasing the expression of pain-related peptides in response to inflammation in local tissues, including IVDs [3]. The neurotrophin family, as represented by nerve growth factor (NGF), contributes to sensitization and nerve ingrowth in IVD tissues [3,6]. Furthermore, the neurotrophin family, including NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3 and its receptor, has been shown to be present in the IVD; its expression is elevated in degenerated and painful discs[7,8].

GDNF (a representative molecule of GFLs), a member of the transforming growth factor-beta (TGF-β) family [9] that is expressed in a wide variety of tissues during development, regulates neuronal gene expression through retrograde transport to the cell soma [9-11]. GDNF basically signals through the complex of the rearranged during transfection (RET) receptor and a member of the GDNF family receptor (GFR) family of glycosyl phosphatidyl-inositol-linked cell surface proteins (GFRα1), which act as ligand binding domains [9]. GDNF, similarly to the neurotrophin family, is known to promote nerve outgrowth and regeneration [12]. GDNF was recently reported to be associated with inflammatory

hyperalgesia [13]. Despite having properties similar to the neurotrophin family, the expression of GDNF family ligands in IVD tissues remains unknown. We hypothesized that GDNF and its receptors are expressed by human IVD cells and that the expression of GDNF is associated with the IVD degeneration that is responsible for discogenic low back pain.

The purpose of this preliminary study was (1) to representatively examine the expression of GDNF among the GFLs and its receptors GFRα1 and RET in human IVD cells and to evaluate the effect of a proinflammatory cytokine on the expression of GDNF and its receptors by human IVD cells, and (2) to immunohistochemically examine those expressions in human IVD tissues with different grades of degeneration.

METHODS

Human IVD tissues and cell isolation

Institutional Review Board (IRB) approval was obtained for this study. Human IVDs were obtained with informed consent from surgical specimens (3 men, 3 women, 34-81 years of age [Average 61 years-old], Pfirrmann's classification[14]: MRI grades II-V). Human IVD cells were cultured in monolayer after separately isolated from both nucleus pulposus (NP) and anulus fibrosus (AF) tissues by sequential enzyme digestion as previously reported[15].

Immunohistochemistry (Immunofluorescence) of human IVD cells

Immunofluorescence of AF and NP cells was performed as previously reported[15]. Briefly, the cells were incubated with a mouse monoclonal antibody raised against GDNF (sc-13147, 1:500, Santa Cruz Biotechnology, Dallas, TX USA), a rabbit polyclonal antibody raised against GFRα1 (sc-10716, 1:500, Santa Cruz Biotechnology), and a rabbit polyclonal antibody raised against RET (sc-9040, 1:500, Santa Cruz Biotechnology). A secondary Alexa 488-conjugated anti-mouse or anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR USA)

antibody was applied for 3 hours. The nuclei were stained with propidium iodide (Molecular Probes). Samples were imaged using confocal microscopy (Fluoview FV1000; Olympus, Tokyo, Japan). Mouse and rabbit IgG serum (DakoCytomation, Glostrup, Denmark) were used as isotype controls.

Western blotting

Cell lysates (containing 20 μg protein) of the NP and the AF cells monolayer-cultured were analyzed with Western blots under reducing conditions as previously reported[16]. The same primary antibodies listed above for the immunofluorescence were used. β-actin served as a loading control for the Western blot assay.

RNA isolation

Total RNA was isolated from human IVD cells in monolayer culture using Isogen (NipponGene, Toyama, Japan), according to the manufacturer's instructions. Total RNA was reverse-transcribed using the 1st strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) with the DNA thermal cycler (Veriti, Applied Biosystems, Foster City, CA USA), according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (PCR)

The expression levels of GDNF (Hs01931883 s1, TaqMan Gene Expression Assay, Applied Biosystems), GFRα1 (Hs00237133 m1, Applied Biosystems), and RET (Hs01120030 m1, Applied Biosystems) were quantified using real-time PCR with TaqMan Gene Expression Assays (Applied Biosystems), employing the primer pairs for TaqMan genomic assays. The assay was calibrated using 18S ribosomal RNA (Hs99999901 s1) as an internal control. To determine the expression of GDNF, GFRα1, and RET, the resultant cDNA (three replicates) was amplified for the target genes. The cycle used a 15-second denaturation at 95°C and 1-

minute annealing and extension at 60°C, utilizing the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative expression of GDNF, GFRα1 and RET was calculated using the comparative threshold (Ct) method [17].

Effect of interleukin-1β on the gene expression of GDNF and its receptors

To examine the effect of proinflammatory cytokines on the mRNA expression of GDNF and its receptors, human IVD cells from both the AF and NP were cultured in the presence of IL-1β (0.1,1.0 and 10 ng/ml) for 48 hours after serum starvation. The mRNA expression of GDNF and its receptors were quantified as described above.

Immunohistochemistry of human IVD tissues

Human IVDs obtained from spine surgeries were divided into two groups, the early stage of degeneration (MRI Pfirrmann's classification[14]: grades II and III, n=15 [9 men, 6 women, 38 – 69 years-old, average age: 55.4 years-old]) and advanced stage of degeneration (MRI Pfirrmann's classification[14]: grades IV, n=15 [5 men, 10 women, 60 - 85 years-old, average age: 73.7 years-old]), according to the extent of disc degeneration evaluated by MRI. Immunohistochemistry of human NP and AF tissues was performed as previously reported[15]. The same primary antibodies (1:500) as the immunofluorescence study (except for anti-GDNF rabbit polyclonal antibody [sc-328, 1:500, Santa Cruz Biotechnology]) were used. Five views of each section at 200× magnification were randomly captured using a digital camera and immuno-positive or -negative cells were manually counted. The immunopositive cells were classified as slightly positive (1+) or strongly positive (2+) according to the intensity of staining as previously reported[16].

Statistical Analysis:

The data are expressed as the mean ± standard error. One-way analysis of variance (ANOVA) was used to assess the effects of culture conditions. Post hoc analyses were

performed using Fisher's least significant difference (LSD). For the histological evaluation of the expression of each protein, statistical differences between the early degenerated stage and the advanced degenerated stage groups were determined using the unpaired Student t-test. All statistical analyses were performed using IBM SPSS Statistics (IBM Japan, Tokyo). Significance was accepted at $P<0.05$.

RESULTS

Fluorescent immunohistochemical analysis of human IVD cells

Immunoreactivity directed against GDNF, GFRα1 and RET were clearly identified in human AF and NP cells cultured in monolayer. Confocal images revealed that immunoreactivity to GDNF, GFRα1 and RET was found in cell membranes and cytoplasm of both AF and NP cells (Fig. 1A-F). The localization of GDNF and its receptors did not differ between AF and NP cells. No immunoreactivity was found in the isotype controls (Fig. 1G).

Western blot analysis of human IVD cells for GDNF and its receptors

Western blot analysis identified a single band directed against GDNF (38 kDa, Fig. 2A), GFRα1 (67 kDa, Fig. 2B), and RET (80 kDa, Fig. 2C) in protein extracts from both AF and NP cells. The expression level of GDNF and GFRα1 in NP cells was higher than that in AF cells; however no significant change was found on RET expression between two types of cells. β-actin (42 kDa, Fig. 2D) expression was similar between AF and NP cells.

Detection of mRNA expression of GDNF and its receptors by human IVD cells

The expression levels of GDNF and its receptors (GFRα1 and RET) were quantified using real-time PCR. mRNA expressions of *GDNF, GFRα1* and *RET* were clearly identified in human AF and NP cells (Fig. 3A-C). Although the mRNA expressions of *GDNF*, *GFRα1* and *RET* were higher in NP cells compared to those in AF cells (relative expression of NP cells

against AF cells: *GDNF* 4.13 ± 2.61, *GFRα1* 2.86 ± 0.97, *RET* 1.38 ±0.57, respectively), statistical significance was not achieved (P=0.32, 0.12, 0.56, respectively).

Effect of interleukin-1β on the gene expression of GDNF and its receptors

The mRNA expression of *GDNF* by AF cells was stimulated by IL-1β treatment dose-dependently (relative expression against control: IL-1β 0.1 [ng/ml]: 2.24 ± 0.64; 1.0 [ng/ml]: 5.34 ± 1.11, P<0.05, 10 [ng/ml] 6.57 ± 2.56, P<0.05, vs. control) (Fig. 4A).Similarly, the mRNA expression of *GDNF* in NP cells was also upregulated by stimulation with IL-1β in a dose-dependent manner. A significant increase of *GDNF* expression by NP cells was found by stimulation with 10 ng/ml of IL-1β (relative expression against control: IL-1β 0.1 [ng/ml]: 3.06 ± 1.40, 1.0 [ng/ml]: 3.94 ± 1.41, 10 [ng/ml]: 4.84 ± 1.25, P<0.05, vs. control) (Fig. 4A).

There was no significant effect of IL-1β on the mRNA expression of *GFRα1* and *RET* by both AF and NP cells (Fig. 4B and C).

Immunohistochemical expression of GDNF and its receptors in human IVD tissues at different stages of degeneration

Immunoreactivity directed against GDNF, GFRα1 and RET was clearly found in human IVD cells in AF and NP tissues at both early and advanced stages of degeneration (Fig. 5). Immunoreactivity for GDNF was found around the nuclei of spindle-shaped cells in the AF region (Fig. 5A, D) and in chondrocyte-like cells in the NP region (Fig. 5G, J). Intense immunoreactivity for GDNF was identified in chondrocyte-like cells with cluster formation in advanced stages of degeneration (Fig. 5J).

GFRα1 and RET immunopositive cells were also found in the AF (Fig, 5B, C, E, F) and NP (Fig. 5H, I, K, L) regions at both early and advanced stages of degeneration.

The percentages of GDNF-immunopositive cells in the AF and NP in the advanced degenerated stage (AF: 71.04 ± 1.51%, NP: 69.95 ± 2.01%) were significantly higher than those in the early degenerated stage (AF: 61.20 ± 3.91%, NP: 63.13 ± 2.63%, $P<0.05$, respectively, Fig. 6A). Interestingly, there was a significant increase of 2+ immunopositive cells in the advanced degenerated stage (AF: 19.3 ± 2.65%, NP: 22.36 ± 3.93%) compared to those in the early degenerated stage (AF: 6.73 ± 1.51%, NP: 9.18 ± 1.86%, $P<0.01$, respectively). There was no significant difference in the percentage of GFRα1-immunopositive cells between the early degenerated stage (AF: 25.70 ± 4.24%, NP: 28.33 ± 4.55%) and advanced degenerated stage (AF: 24.71 ± 4.24%, NP: 19.43 ± 2.63%) (Fig. 6B). No significant differences in the percentage of RET immunopositive cells were also seen between the early degenerated stage (AF: 32.91 ± 5.23%, NP: 36.52 ± 4.99%) and advanced degenerated stage (AF: 30.00 ± 4.11%, NP: 32.69 ± 5.29%) (Fig. 6C).

DISCUSSION

To mimic the proinflammatory cytokine-rich microenvironment of degenerated human IVDs [18], human IVD cells in our study were stimulated by IL-1β *in vitro*. Our results showed that IL-1β significantly elevated the mRNA expression of *GDNF*. Abe and colleagues showed that IL-1β and TNF-α significantly upregulated *NGF* mRNA expression by human IVD cells [7]. Similarly, the expression of BDNF and NT-3 were upregulated by the IL-1β stimulation of human IVD cells[8,19]. Our study also showed that the percentage of immunopositive cells for GDNF in advanced stages of degeneration was significantly higher than that in early stages. Our results, and those of previous studies, suggest the possibility that the expression of NFs, including GDNF, can be regulated by proinflammatory stimuli in the microenvironment of degenerated human IVDs.

Previous studies have shown the association between inflammation and GDNF expression. GDNF expression was upregulated *in vitro* by inflammatory cytokines, such as TNF-α and IL-1β, in astrocytes [20]. Immunohistochemical analysis showed that the expression of GDNF was upregulated in colonic epithelial cells of the inflammatory bowel disease patients [21]. GDNF has been shown to be associated with sensitization of nociceptors by upregulating transient receptor potential cation channel subfamily V member 1 (TRPV1) expression by dorsal root ganglion (DRG) neurons during inflammation [22] and by enhancing the release of calcitonin gene-related peptide (CGRP) from sensory neurons [23]. More recently, Jung and colleagues reported that the intradiscal injection of complete Freund's adjuvant significantly upgraded GDNF expression in the DRGs and thalamus of rats [24]. Considering these previous reports, we speculated that the upregulation of GDNF in degenerated human IVD cells might contribute to the transmission of inflammatory nociceptive pain.

The expression of not only neurotrophins, but also neurotrophin receptors, by IVD cells, as previously reported [7,19], suggests an autocrine or paracrine role for these molecules in regulating the biology of IVDs in addition to a neurotrophic function. Iannone and colleague reported that NGF and its high-affinity receptor tropomyosin receptor kinase A (TrkA) are expressed by human articular chondrocytes and that their expression increases in osteoarthritic cartilage according to the degree of tissue injury [25]. Jiang and colleague showed that NGF signaling is a contributing factor to articular cartilage degeneration in osteoarthritis, which likely targets a specific subpopulation of progenitor cells, affecting their migration and increasing proteoglycan release and matrix metalloproteinases (MMPs) expression [26]. The results of our study also showed that GFRα1 and RET were constitutively expressed in human IVDs. Our *in vitro* study showed that, in contrast to GDNF, the mRNA expression of GFRα1 and RET was not influenced by pro-inflammatory cytokine stimulation. Indeed, the expression of both GFRα1 and RET was identified in human IVD tissues, although no significant differences in the incidence of their immunopositive cells were found between early and advanced stages of degenerated tissues.

Therefore, we speculated that GDNF might also have an autocrine and/or paracrine role in bioactivities, including the matrix metabolism of IVD cells through the GFRα1/RET signaling pathway that are relevant to IVD degeneration. Further studies to examine the autocrine effect of GDNF on disc metabolism and inflammatory responses are needed.

A limitation of this study is that human IVD samples with different stages of degeneration were obtained from spine surgeries and utilized in an *in vitro* study; therefore, a potential bias about the expression of GDNF and its receptors and responses to IL-1β stimulation might exist. Although this *in vitro* culture system cannot reflect the course of human disc degeneration and discogenic pain, the results of this study may contribute to the understanding of cellular responses.

In conclusion, the results of this study for the first time demonstrated that GDNF and its receptors were constitutively expressed in the human IVD at both protein and mRNA levels. The gene expression of GDNF by AF and NP cells was significantly upregulated by treatment with IL-1β. We can speculate that the expression of GDNF is upregulated in degenerated painful IVD tissues, a proinflammatory-rich microenvironment. Because GDNF is one of the major NFs and has been reported to be strongly associated with pain transmission, GDNF might play a key role in pain generation and transmission of discogenic pain.

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Fig. 1

Immunohistochemical staining for glial cell-line derived neurotrophic factor (GDNF), GDNF family receptor (GFR) α1 and rearranged during transfection (RET) receptors in cultured human anulus fibrosus (AF) and nucleus pulposus (NP) cells. AF: A-C, NP: D-F; isotype controls: G. Samples were imaged using confocal microscopy. Nuclei are stained with propidium iodide (red). Scale bar: 20 µm.

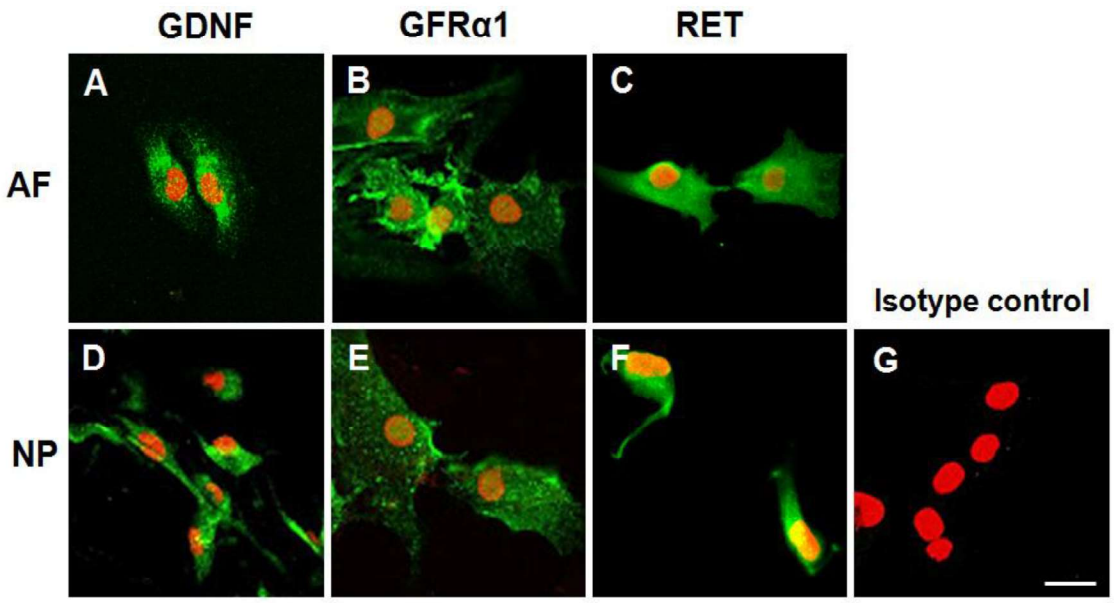

Fig. 2

Western blots for glial cell-line derived neurotrophic factor (GDNF)**(A)**, GDNF family receptor (GFR) α1 **(B)** and rearranged during transfection (RET) **(C)** receptor in cultured human anulus fibrosus (AF) and nucleus pulposus (NP) cells. A single band directed against each antibody was detected. β-actin **(D)** served as a loading control.

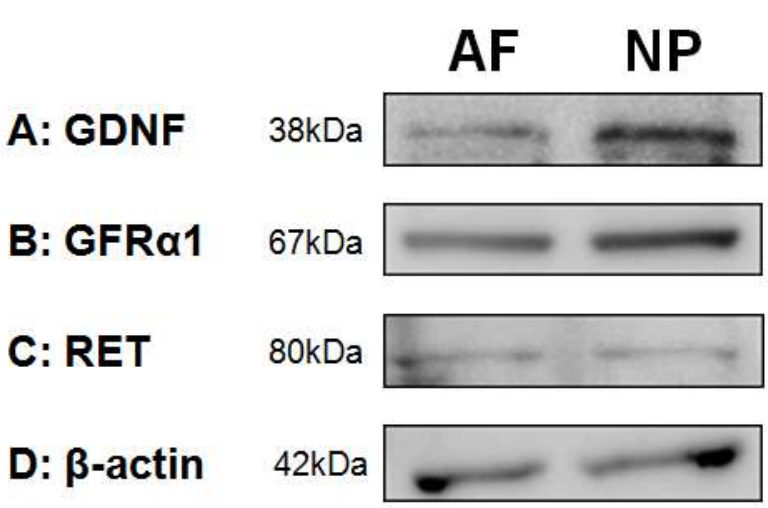

Fig. 3

Detection of mRNA expression by quantitative real-time polymerase chain reaction (PCR) of glial cell-line derived neurotrophic factor (GDNF) **(A)**, GDNF family receptor (GFR) α1 **(B)** and rearranged during transfection (RET) receptor **(C)** in human annulus fibrosus (AF) and nucleus pulposus (NP) cells.

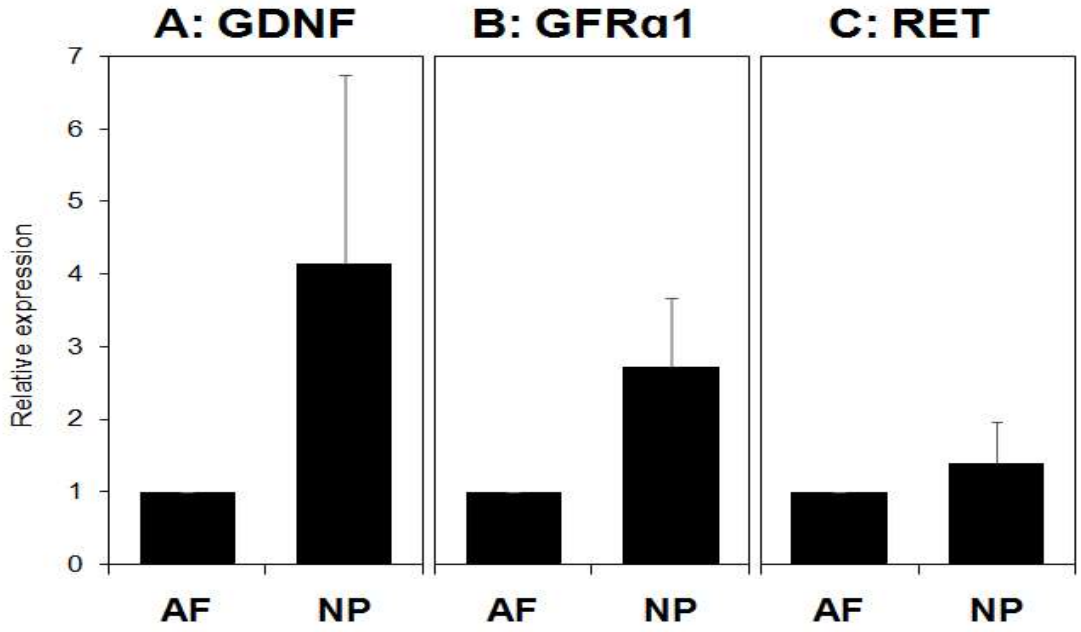

Fig. 4

Effect of interleukin-1 beta (IL-1β) on mRNA levels of glial cell-line derived neurotrophic factor (GDNF) **(A)**, GDNF family receptor (GFR) α1 **(B)** and rearranged during transfection (RET) receptor **C)** by human anulus fibrosus (AF) and nucleus pulposus (NP) cells. IL-1β: Interleukin-1β, Cont.: Control group, *p<0.05, vs. control.

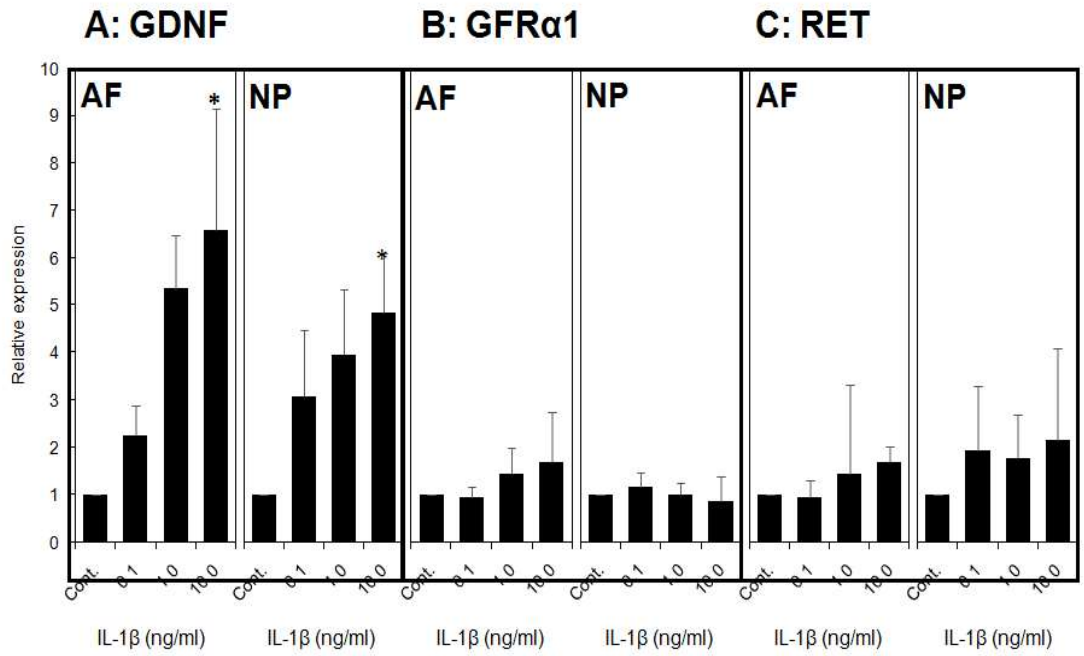

Fig. 5

Immunohistochemical staining for glial cell-line derived neurotrophic factor (GDNF) (A, D, G, J), GDNF family receptor (GFR) α1 (B, E, H, K) and rearranged during transfection (RET) receptor (C, F, I, L) in human intervertebral disc (IVD) tissues (anulus fibrosus: AF [A-F]) and nucleus pulposus: NP [G-L]) at early and advanced stages of degeneration. Scale bar: 20 μm.

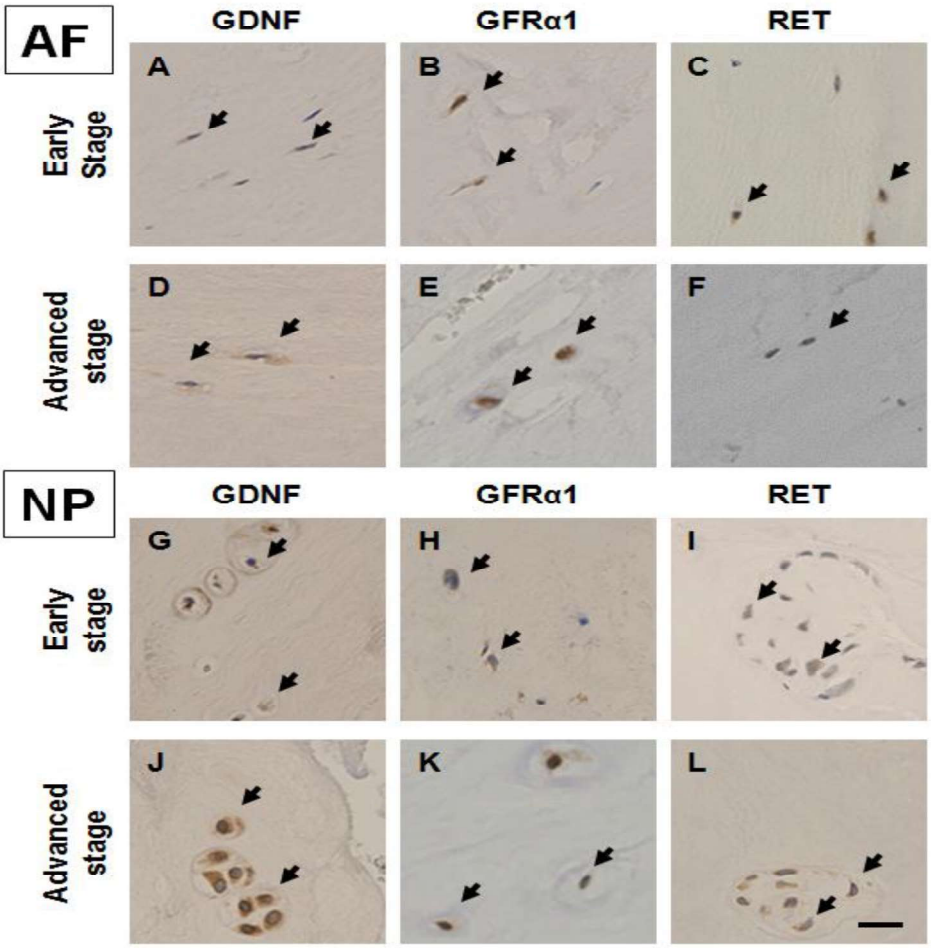

Fig. 6

Percentage of immunopositive cells for glial cell-line derived neurotrophic factor (GDNF) (A), GDNF family receptor (GFR) α1 (B) and rearranged during transfection (RET) receptor (C) in the human IVD tissues of early and advanced stages of disc degeneration. anulus fibrosus: AF, nucleus pulposus: NP. *P<0.05, **P<0.01 between early and advanced stages of degeneration.