Growth Differentiation Factor-6 Attenuated Pro-inflammatory Molecular Changes in the Rabbit Anular-puncture Model and Degenerated Disc-induced Pain Generation in the Rat Xenograft Radiculopathy Model

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INTRODUCTION: During intervertebral disc (IVD) degeneration, the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), increases levels of vascular endothelial growth factor (VEGF) and nerve growth factor (NGF), which promote angiogenesis and innervation resulting in pain (1,2). In dorsal nerve ganglion (DRG) neurons, VEGF and NGF can increase the expression of the nociceptive neuropeptides calcitonin gene-related peptide (CGRP) and Substance P, and also activate microglia; these changes are implicated in neuropathic pain (3,4). Consequently, these results suggest a structural and biological role of inflammation and pain in degenerated disc disease. Growth differentiation factor 6 (GDF6/Bone morphogenetic protein 13; BMP13), plays an essential role in skeletal development (5). The intradiscal administration of GDF6 in an *in vivo* sheep model resulted in preventing loss of disc height, increasing proteoglycan content and collagen synthesis, and maintaining cell number in the IVD (6). However, it is unknown if GDF6 affects pain generation in degenerative discs. In this study, we hypothesized that the structural modification of discs by GDF6 will be associated with the relief of pain induced by degenerated discs. To prove this hypothesis, the two-step disc xenograft radiculopathy model using rabbits and nude rats was utilized and the effects of GDF6 on the inflammatory status of rabbit degenerated discs and pain behavior in the rat radiculopathy model were compared.

METHODS: The study protocol was approved by the Institutional Animal Care and Use Committee.
Rabbit anular-puncture disc degeneration model and the injection of GDF6: Under general anesthesia, lumbar IVDs of female New Zealand white rabbits (n=14) were exposed and the anulus fibrosus (AF) of two noncontiguous discs (L2/3 and L4/5) was punctured by an 18-gauge needle. Four weeks after the initial puncture, either vehicle (phosphate-buffered saline (PBS); 10 µL per disc) or GDF6 (100 µg in 10 µL PBS per disc; PEPROTECH, Rocky Hill, NJ, USA) was injected into the center of the nucleus pulposus (NP) using a 26-gauge needle.
Gene expression analysis (6 rabbits): Total RNA in NP and AF tissues was extracted by bead disruption and Qiazol with further purification using the RNeasy MiniElute Cleanup Kit (Qiagen). Whole transcriptome cDNA libraries were synthesized using the QuantiTect Whole Transcriptome kit (Qiagen). After preamplification, (SsoAdvanced™ PreAmp Supermix, Biorad) gene expressions of aggrecan (ACAN), collagen-II (Col-II), matrix metalloproteinase-3 (MMP-3), IL-1β, IL-6, TNF-α, VEGF, prostaglandin-endoperoxide synthase 2 (PTGS2), and NGF were analyzed using quantitative real-time polymerase chain reaction (PCR) with standards. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.
Nude rat disc xenograft radiculopathy model: To assess mechanical allodynia, female nude rats (NIH-Foxn1^{rnu} n=16) were used for this study. Based on the methods of Kawakami et al. (7), the right L5 DRG was exposed via partial laminectomy and facetectomy under general anesthesia. Degenerated NPs from punctured/injected IVDs (L2/3 or 4/5) of anular-punctured rabbits (PBS; 4, GDF6; 4) were placed on DRGs as xenografts.
Behavioral assessment for mechanical allodynia: Mechanical allodynia was evaluated in nude rats using the 50% paw withdrawal threshold response to mechanical stimulation by Von Frey hair filaments of both ipsilateral and contralateral hind paws.
Immunohistochemistry (IHC) of DRGs**:** The expression of ionized calcium binding adaptor molecule-1 (Iba-1; a microglia/macrophage specific calcium binding protein), and CGRP (a pain-related neuropeptide) were analyzed in DRG neurons of eight rats in each group. The numbers of Iba-1 positive cells were quantified and expressed as cells/mm^2. The number of CGRP-positive and -negative neurons in DRGs was counted and the results are expressed as percentage of CGRP-positive neurons.
Statistical analyses: All data are expressed as mean and standard error (SE). The data were statistically analyzed using One-way repeated analysis of variance (ANOVA, von Frey test and gene expression) or unpaired t-test (IHC). P values less than 0.05 are regarded as statistically different.

RESULTS: The effects of GDF6 on gene expression *(Fig. 1):* In the NP, genes expressions of inflammatory cytokines (IL-1β, IL-6, TNF-α) and pain-related molecules (VEGF, PTGS2, NGF) in the GDF6 group were significantly lower than those in the PBS group (P<0.05). In the AF, there were no significant differences in gene expressions between the two groups.
Mechanical allodynia *(Fig. 2)*: In the PBS group, the withdrawal threshold continued to reduce until day 10 after surgery, but had largely recovered at 14 days. On the other hand, in the GDF6 group, this reduction recovered from day 10 and had essentially returned to baseline by day 14. Significant differences in the withdrawal threshold between the groups were observed on both days 10 and 14 (P<0.05).
Expression of Iba-1 and CGRP in DRGs *(Fig. 3A, B)*: The average number of Iba-1-positive microglia/mm^2 in the GDF6 group was significantly lower than that in the PBS group (-40 %, P< 0.05). The average percentage of CGRP-positive neurons in the GDF6 group was also significantly lower compared to the PBS group (-36 %, P< 0.01).

DISCUSSION: The first-step experiment in the rabbit annular puncture model demonstrated the significant inhibitory effects of GDF6 on gene expressions of various inflammatory/pain-related molecules in rabbit degenerated discs. Importantly, in the subsequent rat xenograft radiculopathy model, mechanical allodynia induced by GDF6-treated rabbit degenerated NPs was significantly lower than that induced by PBS-treated rabbit degenerated NPs. The decreased inflammatory condition, supported by gene expression analyses, corresponded with less pain generation in the rat radiculopathy model. The pain status in these rats was further supported by the immunohistochemistry results (low Iba-1 and CGRP staining) in DRGs of the GDF6-injected IVD transplanted group. The results suggest that pain generation induced by molecules released by degenerated discs may be ameliorated by treatment with GDF6 through the inhibition of inflammatory/ pain-related molecules. The study provides preclinical evidence that GDF6 injection may be effective in humans.

SIGNIFICANCE: GDF6 injection into the IVD may be a therapeutic candidate to attenuate degenerative IVD-induced pain.

FIGURES:

Fig. 1

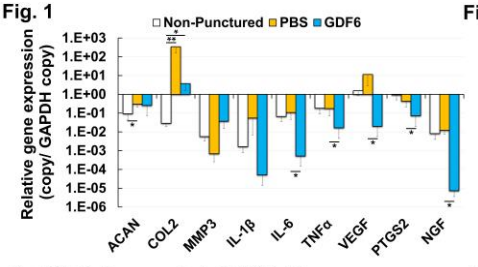

Fig. 1. Relative Gene expression to GAPDH in NP
Data are expressed as mean and standard error (SE). *P< 0.05; **P< 0.01

Fig. 2

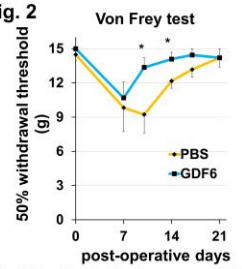

Fig. 2. Von Frey test for the assessment of mechanical allodynia.

Fig.3 A

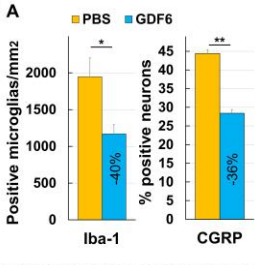

Fig. 3 A. The number of Iba-1 positive microglias and % CGRP positive neurons

Fig.3 B

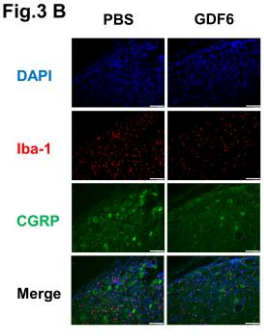

Fig. 3 B. IHC for Iba-1 and CGRP on DRG.
Bar scale was 100 µm