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Differential expression of extracellular-signal-regulated kinase 5 (ERK5) in normal and degenerated human nucleus pulposus tissues and cells

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ABSTRACT

Extracellular-signal-regulated kinase 5 (ERK5) is a member of the mitogen-activated protein kinase (MAPK) family and regulates a wide variety of cellular processes such as proliferation, differentiation, necrosis, apoptosis and degeneration. However, the expression of ERK5 and its role in degenerated human nucleus pulposus (NP) is hitherto unknown. In this study, we observed the differential expression of ERK5 in normal and degenerated human nucleus pulposus tissues by using immunohistochemical staining and Western blot. Treatment of NP cells with Pro-inflammatory cytokine, TNF-α decreased ERK5 gene expression as well as NP marker gene expression; including the type II collagen and aggrecan. Suppression of ERK5 gene expression in NP cells by ERK5 siRNA resulted in decreased gene expression of type II collagen and aggrecan. Furthermore, inhibition of ERK5 activation by BIX02188 (5 μM) decreased the gene expression of type II collagen and aggrecan in NP cells. Our results document the expression of ERK5 in degenerated nucleus pulposus tissues, and suggest a potential involvement of ERK5 in human degenerated nucleus pulposus.

1. Introduction

The intervertebral disc (IVD) is a fibrocartilage load-bearing tissue that lies between vertebrae. It plays an essential role in supporting loading of the spine and in providing motion and flexibility of the spine. Degeneration of the IVD is commonly associated with low back and cervical pain, particularly in people with ageing and occupational exposures [1]. The IVD is composed of two parts: the central gelatinous nucleus pulposus (NP) and the outer lamellar annulus fibrous (AF) [2]. The nucleus pulposus is composed of chondrocyte-like nucleus pulposus cells that produce type II collagen and proteoglycan (i.e. aggrecan), and reside in an environment with lack of blood supply [2]. Increased degradation of aggrecan and changes in extracellular matrix (ECM) content are frequently observed in NP tissues under pathological conditions, which are accompanied with increased necrotic cell death, leading to increased disc degeneration, stiffness and back pain [3]. Understanding the molecular and cellular regulation of NP cells will gain insights into the pathogenesis of IVD degeneration and help to identify new approaches to facilitate restoring the structure and function of the IVD.

ERK5 (extracellular-signal-regulated kinase 5), a recently discovered member of the MAPK family [4–6], is expressed in a variety of tissues, and regulated by growth factors, cytokines and oxidative stress [7–9]. Interestingly, TNF-α expression has been localized to cells in both the AF and NP and in herniated disc tissue [10,11]. In contrast to other cytokines whose production is limited to herniated disc tissue, TNF-α expressing cells are present within degenerated nonherniated NP, and associated with age and the degree of disc degeneration [12]. Furthermore, TNF-α has been shown to contribute to the pathophysiology of NP-induced nerve root injury and the initiation of pain [13]. This suggests an important role for TNF-α in mediating IVD degeneration and associated morbidity. However, the expression of ERK5 in degenerated human NP cells and its regulation by TNF-α is unknown.

In this study, we report that ERK5 was differentially expressed in human normal and degenerated NP tissues and cells. TNF-α could decrease the gene expression of ERK5, as well as NP marker gene expression of type II collagen and aggrecan. Furthermore, gene silencing of ERK5 and inhibition of ERK5 activity by BIX02188 (5 μM) decreased the gene expression of type II collagen and aggrecan in NP cells.

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2. Materials and methods

2.1. Materials

RNA isolation kits were purchased from TaKaRa (Tokyo, Japan). Western blot kits and immunohistochemical staining were purchased from GE Healthcare (Piscataway, NJ, USA). Antibodies to ERK5, beta-actin and IgG were purchased from Cell Signalling Technology (Danvers, MA). BIX02188 was purchased from Abcam (Cambridge, MA).

2.2. Isolation and passage of nucleus pulposus cells

Normal nucleus pulposus tissues were obtained from seven cases of scoliosis that underwent orthopedic surgery (four cases aged 13 years, three case aged 15 years). Degenerated nucleus pulposus tissues were obtained from five cases of disc degeneration that underwent orthopedic surgery. To obtain NP cells, the intervertebral disc was removed and soaked in saline containing penicillin–streptomycin antibiotics for 10 min, and the nucleus pulposus tissues were gently separated from the disc using a curette, followed by 3 to 4 washes with saline until no blood was visible. Nucleus pulposus tissues which showed normal appearance of a peripheral white fibrous ring surrounding the central jelly-like nucleus pulposus structure were then cut into $1 \times 1 \times 1\ mm^3$ pieces with ophthalmic scissors, and placed in a 100 ml beaker filled with 10 ml of 2% collagenase II and stirred for 60 min. The completely digested tissues were centrifuged at 1000 r/min for 10 min. The supernatant was aspirated and the cells were dispersed using 1 ml DMEM containing 10% fetal calf serum, and cells were then cultured in a T25 tissue culture flask with 6 ml DMEM containing 10% fetal bovine serum at 37 °C, saturated humidity, and 5% CO_2 for three days.

2.3. Immunohistochemical staining of ERK5

NP tissues were embedded using paraffin and cut serially at 5 μm for immunohistochemical staining (IHC). The staining procedure was done following the Cell and Tissue Staining Kits (CTS008, R&D System, USA). Primary antibody to ERK5 was used to stain ERK5 protein expression, and IgG was used as a negative control. Finally, DAB staining was performed using appropriately diluted DAB/metal concentrate (PIERCE).

2.4. Western blot analysis

For each experimental time point, each sample used in this study was homogenized in radio-immunoprecipitation assay (RIPA) buffer supplemented with benzylsulfonyl fluoride (PMSF) on ice for 30 min. The homogenate was centrifuged at 12,000×g for 15 min at 4 °C. Collected the supernatants to new tubes. Proteins were separated on 8% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. After transfer to the PVDF membranes, filters were blocked for 1 h at room temperature in 3% bovine serum albumin (BSA) and then incubated with the corresponding primary antibody at 4 °C over night. After being washed with tris-buffered saline and Tween 20 (TBST), filters were incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibodies and then detected using BeyoECL plus and chemiDoc XRS+ Imaging System.

2.5. Gene knockdown of ERK5 by siRNA

Cells were transfected with ERK5 siRNA (sequence: CACGACAA CATCATCGCCA) or scramble siRNA (sequence: TTCTCCGAACGTGTC ACGT) at a final concentration of 30 nM using Lipofectamine 2000. Six hours after transfection, the medium was replaced with complete growth medium. Total RNA was isolated after 48 h and subjected to real time RT-PCR analysis.

2.6. Real time PCR analysis of type II collagen, aggrecan, ERK5 gene expression

Fluorescence quantitative PCR (SYBR Green) was performed to detect the expression of type II collagen, aggrecan, and ERK5 gene expression. Primer express 2.0 software was used for primers design. All primer sequences are listed in Table 1.

2.7. TNF-α treatment of the NP cells

NP cells were plated in 6-well plates, when the cells reached 90–95% confluent, cells were stimulated with 10 ng/ml TNF-α. Fluorescence quantitative PCR (SYBR Green) was performed to detect the expression of type II collagen, aggrecan, and ERK5 gene expression at 0, 6, 12, 24, 36, and 48 h after TNF-α intervention.

2.8. ERK5 inhibition by BIX02188

NP cells were treated BIX02188 (5 μM) to inhibit ERK5 activity for 48 h [14], and real time RT-PCR was performed to analyze the mRNA expression of type II collagen and agreccan.

3. Results

3.1. Differential expression of ERK5 in normal and degenerated human nucleus pulposus tissues

Using immunohistochemical staining, we first examined the expression of ERK5 in NP tissues and cells. As shown in Fig. 1, ERK5 protein expression was detected in degenerated (Fig. 1C) and normal NP tissues (Fig. 1E) using anti-ERK 5 antibodies but not with anti-IgG control (Fig. 1A). ERK5 protein expression was also detected in degenerated NP cells (Fig. 1D) and normal NP cells (Fig. 1F), but not with anti-IgG control (Fig. 1B) by immunohistochemical staining. It appears that there was a decreased level of ERK5 expression in degenerated NP tissues and cells. To quantitatively compare the ERK5 protein expression between normal and degenerated NP tissues, Western blot analysis was performed. The results showed ERK5 expression in degenerated NP tissues was statistically significantly lower than that of the normal NP tissues (Fig. 2A, B).

Table 1
Primer Sequences for PCR.

Primers	Forward	Reverse	Length
ERK5	5′-GTGCCCTATGGCGAATTCAA-3′	5′-GCACGTGTTCCAGTGTGAGG-3′	106bp
COLLAGEN II	5′-TGGTGGCTTCCATTTCAGCT-3′	5′-TGTTCTGGGAGCCTTCCGT-3′	104bp
AGGRECAN	5′-AGCCTGCGCTCCAATGACT-3′	5′-GGAACACGATGCCTTTCACC-3′	103bp
β-actin	5′-GCATGGGTCAGAAGGATTCCT-3′	5′-TCGTCCCAGTTGGTGACGAT-3′	106bp

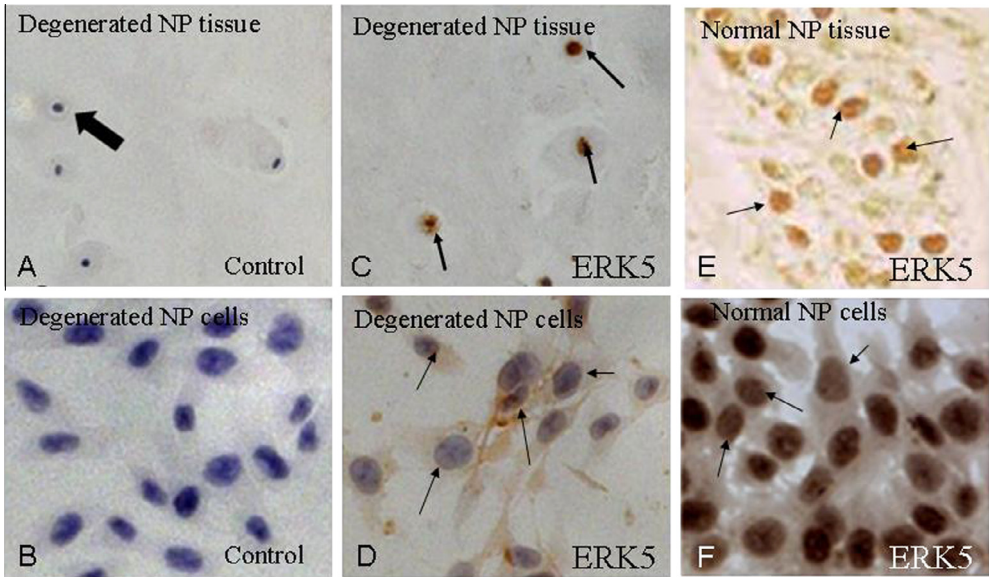

Fig. 1. The control groups showing negative immunohistochemical staining of ERK5 in NP tissues (A) and cells (B) with IgG antibody. Immunohistochemical staining showing positive cytoplasmic signals (claybank) of ERK5 in degenerated NP tissue (C) and cells (D), as well as normal NP tissue (E) and cells (F).

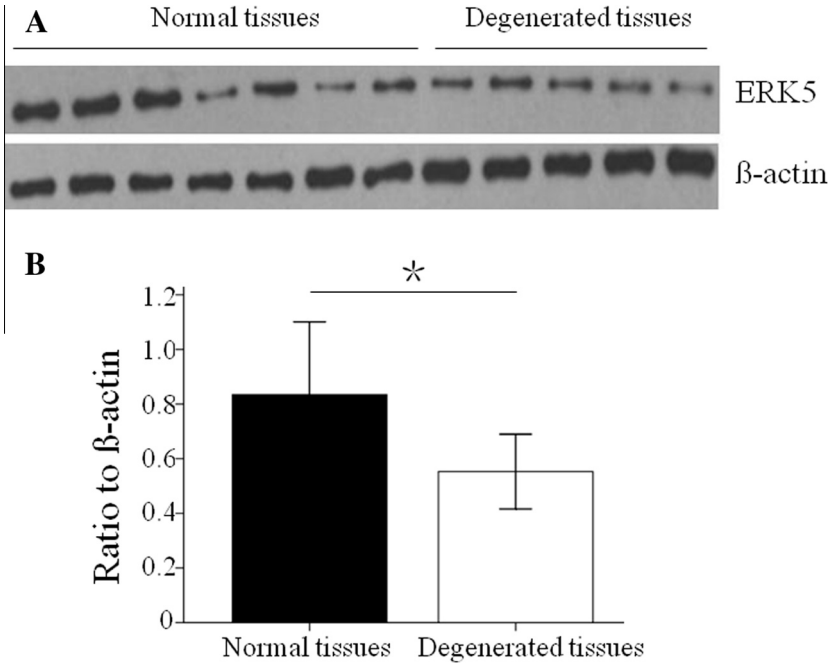

Fig. 2. Western blot analysis (A) and qualitative measurement of ERK5 versus beta actin ratio (B) revealed that there was a significant decrease of ERK5 expression in degenerated NP tissues as compared to normal tissues (*P Value < 0.05).

3.2. TNF-α treatment reduced the gene expression of ERK5, COL2A1 and aggrecan in NP cells

Since TNF-α has been implicated in the degeneration of NP cells, we tested the effect of TNF-α on the gene expression of ERK5, and NP cell maker genes type II collagen and aggrecan. As shown in Fig. 3, the ERK5 mRNA expression was reduced after TNF-α treatment from 6, 12, 24, 36, and 48 h. The reduced expression of ERK5 was also accompanied with the significantly reduced expression of NP cell maker genes type II collagen and aggrecan (Fig. 3).

3.3. siRNA-mediated knockdown of ERK5 resulted in reduced mRNA transcript levels of the NP marker genes type II collagen and aggrecan

To explore the role of ERK5 in NP cells, we employed siRNA-mediated knockdown of ERK5 and examined the mRNA transcript levels of the NP cell marker genes type II collagen and aggrecan. ERK5 mRNA expression was reduced to >50% as compared with the control. Importantly, NP cells transfected with a siRNA sequence targeting ERK5 gene resulted in decreased levels of mRNA transcripts of the NP cell maker genes type II collagen and aggrecan to 49% and 47% respectively, relative to control siRNA treatment (Fig. 4A).

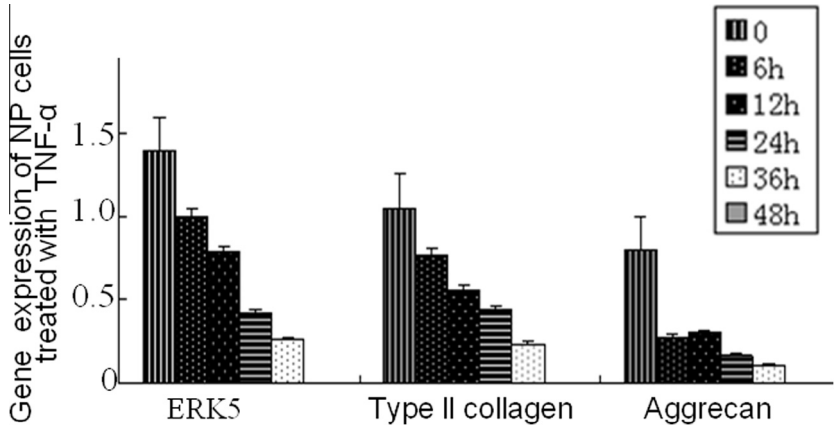

Fig. 3. Real time RT-PCR analysis showing that the mRNA expression of ERK5, type II collagen, aggrecan in NP cells was reduced at 6 h, 12 h, 24 h, 36 h, 48 h after the treatment of 10 ng/ml TNF-α.

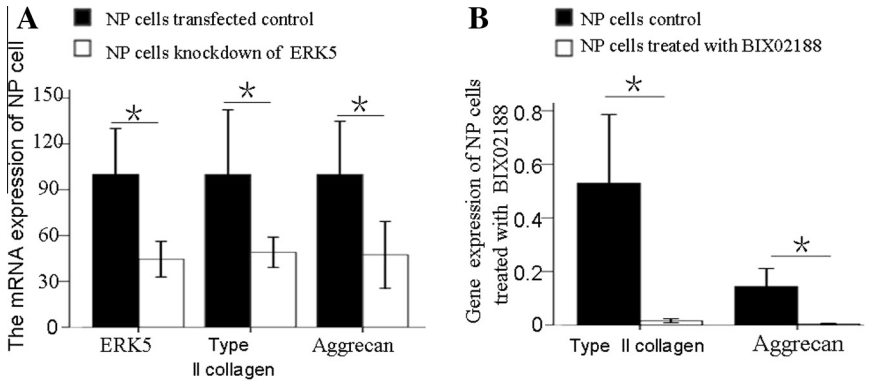

Fig. 4. siRNA silencing of ERK5 gene showed reduced mRNA expression of ERK5 in the normal NP cells. Importantly, NP cells transfected with siRNA sequences targeting ERK5 gene reduced the mRNA transcripts of the nucleus pulposus characteristic maker genes such as type II collagen and aggrecan (*P Value < 0.05) (A). Treatment of NP cells with BIX02188 (5 μM) for forty-eight hours reduced the mRNA expression of type II collagen and aggrecan (*P Value < 0.05) (B).

3.4. NP cells treated with ERK5 inhibitor BIX02188 resulted in reduced mRNA transcript levels of the NP marker genes type II collagen and aggrecan

To explore the role of ERK5 in NP cells, we treated NP cells with ERK5 inhibitor BIX02188 and examined the mRNA transcript levels of the NP cell marker genes type II collagen and aggrecan. After treatment of NP cells by BIX02188 (5 μM) for 48 h, the mRNA expression of type II collagen and aggrecan was significant decreased ($P < 0.05$) (Fig. 4B).

4. Discussion

The main pathologic changes of the degenerated disc include the degradation of proteoglycan and abnormal composition of collagen in extracellular matrix, as well as the significant decrease of water content in the NP tissues. Studies have shown that biomechanics factors [15], genetic factors [16], aging [17] and the unhealthy living habits such as smoking and drinking [18], especially inflammatory factors [15], were among the causes of intervertebral disc degeneration.

TNF-α is a member of the inflammatory with a broad spectrum of bioactivities including cytotoxicity, cell proliferation, growth, and differentiation [12], and TNF-α mediated MAPK pathways were involved in the degeneration of NP tissues [13].

Among the MAPK pathways, ERK5 signaling pathway is one of the members of MAPK family that has been identified recently in the regulation of a variety of cellular processes [9,19], and ERK5 protein expression has been reported in chondrocytic cells [20]. However, the relationship of the ERK5 signaling pathway and NP degeneration has not been previously described, and thus the focus of this study.

In this report, we examined ERK5 expression in human degenerated NP tissues. Immunohistochemical staining and Western blot revealed that ERK5 protein is expressed in the degenerated NP cells and tissues. Western blot revealed that decreased levels of ERK5 in degenerated NP tissues as compared to normal tissues. RT-PCR revealed that TNF-α decreases ERK5, type II collagen and aggrecan gene expression. siRNA-mediated knockdown of ERK5 and inhibition of ERK5 inhibitor BIX02188 resulted in reduced mRNA transcript levels of the NP cells marker genes type II collagen and aggrecan.

According to the results above, we speculated that the status of ERK5 would be changed in the process of NP degeneration. Although studies on NP degeneration showed that the levels of ERK1/2 were steadily increased in NP cells in the process of degeneration induced by TNF-α [13], in our study, the levels of ERK5 were declined. These data suggest that the ERK5 and ERK1/2 signaling pathways may play opposing regulatory roles during NP degeneration induced by TNF-α.

In conclusion, our results suggest a novel role for the ERK5 pathway as an important modulator of human NP degeneration.

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