

Original Research Article

Inhibitory activity of CXCR4 attenuates intervertebral disc degeneration by regulating TXNIP/NLRP3 expression

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Abstract

Purpose: To explore the inhibitory effect and mechanism of action of CXCR4 on intervertebral disc degeneration.

Methods: The expression of CXCR4 was assessed in different degrees of degenerate disc tissues. Nucleus pulposus (NP) cells-induced degeneration were obtained using interleukin 1 beta (IL-1 β) stimulation. CXCR4 inhibitor, AMD 3100, was employed to resist the function of CXCR4, in order to determine whether CXCR4 inhibition acted by targeting TXNIP/NLRP3 axis.

Results: Expression of CXCR4 increased in severely degenerated disc tissues. IL-1 β activated and promoted the progression of intervertebral disc degeneration, but AMD3100 treatment reversed the effects of IL-1 β . Moreover, TXNIP and NLRP3 were significantly suppressed by AMD3100 stimuli.

Conclusion: The results demonstrate that inhibition of CXCR4 prevents NP cells from degradation by regulating TXNIP/NLRP3 signaling pathway, thus indicating that this approach could be effective in the management of intervertebral disc degeneration.

Keywords: Nucleus pulposus cells, intervertebral disc degeneration, CXCR4, Inflammation

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INTRODUCTION

Lumbar disk herniation with low back pain is one of the frequent diseases in spinal surgery [1], resulting from pathological degeneration of the intervertebral disc in biochemical composition and architecture, including metabolic disturbance of the extracellular matrix (ECM), reduced water content as well as loss of the disc height [2]. Nucleus pulposus (NP) cells, as the dominant cell group in the intervertebral disc, solely regulates the hydrologic balance and ECM proportion in the intervertebral disc [3]. Hence, IDD is associated with the inactivation of NP

cells. Persistent chronic inflammatory stimulation and accumulating proinflammatory cytokines have been identified to be responsible for ECM breakdown. The elevation of interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α plays a critical role in IDD [4]. An elaborate understanding of the molecular interplay concerning inflammation and NP cells vitality is needed.

Chemokine CXC receptor 4 (CXCR4) is a member of the G protein-coupled receptor family [5]. Previous studies have reported that CXCR4 increases in degenerated intervertebral discs [6],

inhibits SDF-1/CXCR4/NF- κ B pathway, and reduces inflammation in human degenerative nucleus pulposus cells [7]. Recently, it has been reported that SDF-1 and its receptor CXCR4 induced matrix metalloproteinase expression in cartilage endplate degradation [8], indicating that the crosstalk between SDF-1 and CXCR4 contributes to the development of IDD. However, the molecular regulatory mechanisms of NP cells CXCR4 in IDD remain elusive.

Thioredoxin interaction protein (TXNIP) is an endogenous thioredoxin that inhibits reactive oxygen scavenging protein in a variety of cells, and participates in regulating oxidative stress and inflammatory response of energy metabolism [9]. Repression of TXNIP inhibits myeloperoxidase activity and oxidative stress in nonalcoholic steatohepatitis [10], and the suppression of TXNIP-NLRP3 inflammasome signal pathway alleviates the degeneration of the intervertebral disc [11]. CXCR4 notably regulates neuropathic pain by targeting TXNIP-NLRP3 inflammasome axis [12], suggesting there may be an association between TXNIP and CXCR4 involved in IDD pathologic change. This study desires to confirm the functional and regulatory mechanism of CXCR4 and TXNIP in inflammation and apoptosis of NP cells, and attempts to discover a promising therapeutic target for IDD.

EXPERIMENTAL

Tissue samples

Tissues of human discs were obtained from the patients who underwent discectomy. The procedure was approved by the Ethics Committee of Beijing Jishuitan Hospital and followed the guidelines of Declaration of Helsinki. Informed consent from patients was obtained before sample collection. Twenty patients (mean age, 45; age range, 34 – 58; 12 males, 8 females) donated their degenerative disc tissues. NP non-detached with the endplates was selected for cell isolation. Following the Pfirrmann score of disc degeneration, we divided the samples into mild group (Grade II or III) and severe group (Grade IV or V). Pending tissues were stored in liquid nitrogen until used in the study.

Cell culture and drug treatment

Fresh disc tissues were washed with Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, Keygen, Nanjing, China)

and fragmented. Dissociation was performed using 0.25 % trypsin solution at 37 °C for 20 min. The mixtures were treated with 0.15 % type II collagenase at 37 °C overnight. The cell suspension was transferred onto a cell strainer (100 μ m). Cells were treated with 5 ng/mL IL-1 β (Sigma-Aldrich, St. Louis, MO, USA) to evoke degradation. After the cells were attached to the culture plate, the optimized concentration of AMD 3100 (MedChemExpress, Monmouth Junction, NJ, USA) ranged from 1 to 20 μ g/mL and the optimized stimulus time by different time pots was 3, 6, 12, and 24 h). ATI-2341 (MedChemExpress, Monmouth Junction, NJ, USA) was administered to enhance CXCR4 expression.

Cell viability assay

Cell counting kit 8 (CCK8) assay was performed to determine NP cells' viability, using CCK8 cell viability/cytotoxicity assay kit (C0009, Beyotime, Shanghai, China), following the manufacturer's instructions. NP cells were transferred into 96-well plates at a density of 1×10^4 cells/well. Using different concentrations of AMD 3100 and different treatment timelines, absorbance was measured using a microtiter plate reader (Labsystems Multiskan, MS, Finland) at 570 nm.

Quantitative real-time-polymerase chain reaction (qRT-PCR)

Total RNA of NP cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in conformity with the manufacturer's protocol. RNA was compounded with complementary deoxyribose nucleic acid (cDNA) using PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). Then, CXCR4, TXNIP, type II collagen, TNF- α , IL-6, iNOS, caspase 3/8, and 18S rRNA were evaluated using SYBR PremixEx TaqII kit (RR820A, TaKaRa, Tokyo, Japan). The primers were listed in Table 1 and the 2- $\Delta\Delta$ Ct methods were used to calculate the relative mRNA levels.

Western blot (WB) assay

Disc tissues or NP cells were harvested using protein lysis buffer (Beyotime, Shanghai, China) with phosphatase and protease inhibitors. After violet oscillation and low temperature centrifugation, protein concentration was measured with the enhanced bicinchoninic acid (BCA), Protein Assay Kit (Beyotime, Shanghai, China), and then balanced.

Table 1: Primer sequences used in PCR

Gene		Primer sequence
CXCR4	Forward (5' > 3')	CGTCGTGCACAAGTGGATCT
	Reverse (5' > 3')	GTTTCAGGCAACAGTGGGAAGAAG
Collagen II	Forward (5' > 3')	TGGACGATCAGGCGAAACC
	Reverse (5' > 3')	GCTGCGGATGCTCTCAATCT
Caspase 3	Forward (5' > 3')	GCCATCGTGGCTAAACAGGTA
	Reverse (5' > 3')	GTTGGTGTTCATCCGCTTGC
Caspase 8	Forward (5' > 3')	ATGAACCAGACTGCGTGCCCTG
	Reverse (5' > 3')	AAGAAGAAGACACCACCCC
18S rRNA	Forward (5' > 3')	GTTGGTTTTTCGGAAGTGAAGG
	Reverse (5' > 3')	GTCGGCATCGTTTATGGTGC

Following separation in 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transfer to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), the protein was blocked with 5 % defatted milk and incubated overnight at low temperature with the primary antibodies (anti-CXCR4 (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase3/8 (Abcam, Cambridge, MA, USA, 1:1000), anti-type II collagen (Millipore, Billerica, MA, USA, 1:1000), anti-TXNIP (Abcam, Cambridge, MA, USA, 1:1000), anti-NLRP3 (Abcam, Cambridge, MA, USA, 1:500), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000). After incubation with the secondary antibody (Abcam, Cambridge, MA, USA, 1:2000), immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Foster City, CA, USA).

Enzyme linked immunosorbent assay

NP cells were collected and evaluated by enzyme linked immunosorbent assay (ELISA) using ELISA kit (KeyGEN, Nanjing, China), following the manufacturer's instruction. The absorbance (A) of each well was measured at the wavelength of 450 nm. Then, standard curve was plotted to calculate the sample concentration.

Immunocytofluorescence (IF) staining

The NP cells were washed by phosphate buffered saline (PBS) and fixed with 4% polyformaldehyde for 15 min, then carried out block for 1 h. Cells were incubated overnight at 4 °C with a primary antibody against TXNIP (Abcam, Cambridge, MA, USA, 1:200), followed by Cy3-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA, 1:200) for 1 h. The nuclei were counterstained with DAPI (Beyotime, Shanghai, China, 1:500), and the cells were visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Flow cytometry

Apoptosis evaluation kit (KeyGEN, Nanjing, China) was used to determine the degree of NP cell apoptosis. Following the manufacturer's protocol, Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) were stained with NP cells for 30 min in lucifuge condition. Then cells were sorted and analyzed using a fluorescence-activated cell sorting flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for data analysis. Data are presented as mean \pm standard deviation (SD). Differences between two groups were analyzed by Student's t-test. Comparison between multiple groups was performed using one-way ANOVA followed by Post Hoc Test (Least Significant Difference). Significant difference was set at $P < 0.05$.

RESULTS

Expression of CXCR4 increased in human degenerated discs

Human disc tissues were harvested into protein and RNA, and allowed to perform the detection of type II collagen and CXCR4 expression. Western blotting aims to measure type II collagen and CXCR4 at protein level in different grades of disc degeneration. Besides, RNA levels of type II collagen and HMGB1 were determined using qRT-PCR. Three samples from the mild group and the severe group respectively were randomly selected; the protein level of type II collagen was reduced with the Pfirrmann grades elevation, and CXCR4 reversed the increased expression in the severe group compared with that in the mild group (Figure 1 A). Besides, in RNA level, we also discovered that CXCR4 expression in the severe group was at a high level compared with the mild group (Figure 1 B). Consequently, CXCR4 expression

increased as the disc suffered severe degeneration.

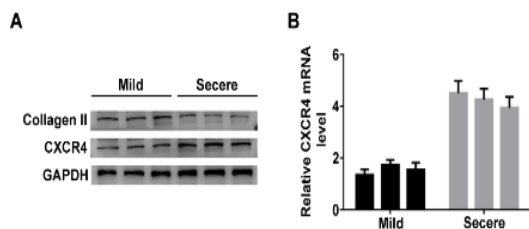


Figure 1: Expression of CXCR4 increased in human degenerated discs. (A) type II collagen and CXCR4 protein expressions in the mild group and the severe group. (B) CXCR4 mRNA level in the mild group and the severe group

Effect of AMD3100 on NP cells viability

The results of CCK8 assay indicate that 10 µg/mL AMD 3100 and 1510 µg/mL ATI-2341 produced the highest cell viability (Figure 2A). However, there was no statistical difference at different time points following AMD 3100 and ATI-2341 administration (Figure 2 B). Therefore, 10µg/ml AMD 3100 was used to treat the stimulated NP cells for 24 h. NP cells *in vitro* evoked degeneration model using IL-1β (10 ng/mL). Type II collagen level was used to estimate the senescent degree of NP cells. CXCR4 expression level was measured both in protein and RNA. It was discovered that IL-1β stimulation successfully induced type II collagen reduction and CXCR4 elevation when compared with those in the control group. Nevertheless, AMD 3100 inhibited CXCR4 expression and prevented type II collagen decline compared to the IL-1β group. Besides, ATI-2341 also enhanced the damaged function of CXCR4 activation (Figure 2 C and D).

CXCR4 inhibition lowered inflammatory effect in senescent NP cells

TNF-α, IL-6 and IL-8 levels were determined by ELISA to assess inflammatory expression, and it was the levels of the above inflammatory cytokines increased in senescent NP cells compared with those in the control group. The results showed that AMD 3100 suppresses inflammation in the NP cell degenerated model. Furthermore, to ascertain whether CXCR4 overexpression promotes inflammatory activity in senescent NP cells, ATI-2341 was used to reinforce the function of CXCR4. The results revealed that CXCR4 overexpression aggravated the inflammatory effect in senescent NP cells (Figure 3 A). Moreover, TXNIP and NLRP3 protein levels were inhibited by AMD 3100 treatment, yet they were elevated in CXCR4

overexpression (Figure 3 B), and immunofluorescence revealed that CXCR4 inhibition reduced TXNIP in NP cells following IL-1β stimulation, but CXCR4 overexpression promoted TXNIP levels in stimulated NP cells (Figure 3 C), suggesting that CXCR4 induced inflammation in NP cells through the regulation of TXNIP/ NLRP3.

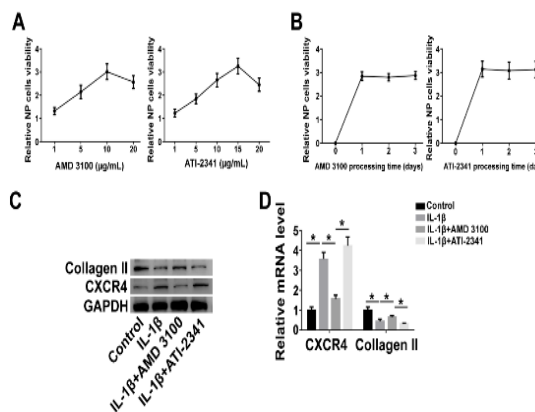


Figure 2: Effect of AMD3100 on NP cell viability. CXCR4 inhibition in NP cells ameliorates degenerative collagen reduction *in vitro*. (A) 10 µg/mL AMD 3100 and 1510 µg/mL ATI-2341 prominently improved the highest cells viability. (B) NP cell viability with AMD 3100 and ATI-2341 treatment of different processing time. (C) ATI-2341 enhanced the damaged function of CXCR4 activation. (D) mRNA levels of CXCR4 and type II collagen in the control, IL-1β, IL-1β +AMD 3100 and IL-1β +ATI-2341 group

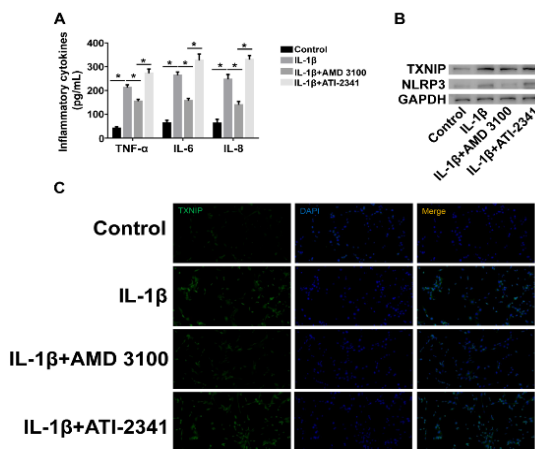


Figure 3: CXCR4 inhibition decreased inflammatory activity in senescent NP cells. (A) CXCR4 overexpression aggravated inflammatory effect in senescent NP cells. (B) TXNIP and NLRP3 protein levels were inhibited in AMD 3100 treatment. (C) IF staining data indicate that CXCR4 inhibition reduced TXNIP in NP cells following IL-1β stimulation, but CXCR4 overexpression enhanced TXNIP levels in stimulated NP cells

Decreased CXCR4 expression reduced apoptosis in senescent NP cells *in vitro*

NP cell apoptosis is considered one of the primary factors of IDD. Hence, the apoptosis-associated proteins were detected in senescent NP cells. The results showed that caspase 3 and caspase 8 decreased significantly following AMD 3100 simulation compared to the IL-1 β group, but caspase 3 and caspase 8 increased remarkably with ATI-2341 treatment (Figure 4 A and B, $p < 0.05$). The flow cytometry results showed that AMD 3100 group exhibited few total apoptosis cells when compared with IL-1 β group; however, the apoptosis cells in ATI-2341 group were more than those in IL-1 β group (Figure 4 C).

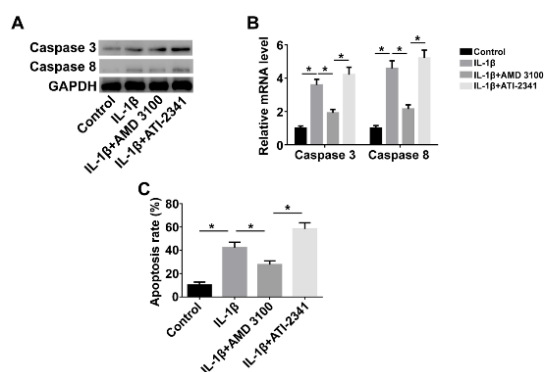


Figure 4: Decreased CXCR4 expression reduced apoptosis in senescent NP cells *in vitro*. (A) Protein levels of caspase 3 and caspase 8 increased remarkably with ATI-2341 treatment. (B) mRNA expressions of caspase 3 and caspase 8 increased remarkably with ATI-2341 treatment. (C) apoptosis ratio in NP cells of four groups

DISCUSSION

Lumbar disc herniation is a common disease in the elderly, which results from IDD in morphological and biomechanical degradation [13]. NP cells maintain intervertebral disc stability and hence produce extracellular matrix and secrete pro-inflammatory cytokines [14,15]. IDD is associated with NP cells undergoing increased cell death and degradation of the extracellular matrix [16]. Studies have shown interest in the pathophysiological process of IDD, including aging [17], constant mechanical loading [18], infection [19] and calcification [20]. However, the complicated mechanism of IDD seems to involve inflammation and apoptosis as widely accepted specific features of the process.

Activation of CXCR4 has been reported to play a positive role in many inflammatory events ranging from recruitment of inflammatory cells to

damaged tissues [21] to differential regulation of expression of inflammatory genes [22]. Previous studies have proved that CXCR4 inhibition can ameliorate autoimmune joint inflammation [23], protect blood-brain barrier integrity and also inhibit inflammatory response after focal ischemia [24]. Therefore, it was speculated that inhibiting the activity of CXCR4 can reduce pro-inflammatory signaling loops directly. Besides, we also desire to probe into the effect of cell apoptosis using the inhibitor of CXCR4. AMD3100, a potent and specific antagonist of CXCR4 was used in this research to inhibit CXCR4 activity. TXNIP, a member of the α -arrestin protein superfamily, expresses in normal tissues and cells, and has been shown to be key to activating NLRP3 inflammasomes by directly interacting with NLRP3 [25].

From the results of the different expressions of CXCR4 gene in both mild and severe group, it is obviously that disc samples with greater degrees of degeneration have higher CXCR4 expression levels and the lower type II collagen expression. With regard to NP cell culture, CCK8 assay was used to explore the optimized concentration and stimulation time of the AMD3100 and ATI-2341; the results indicate that AMD3100 and ATI-2341 have an ability to promote the proliferation of NP cells at an optimized concentration. IL-1 β was widely used for the stimulation of IDD pathophysiology. Hence NP cells degenerated model was established using IL-1 β . It was shown that the inflammation marker levels (TNF- α , IL-6 and IL-8) specifically increased with the IL-1 β stimuli compared with the control, but via inhibition of TXNIP and NLRP3 following AMD 3100 treatment. The pro-inflammatory role of TXNIP and NLRP3 overexpression in NP cells was influenced by the addition of ATI-2341. Recent evidence has suggested that CXCR4 activation is a probable proinflammatory cytokine in the progression of NP cell degeneration and that it is efficiently inhibited by AMD 3100.

Apoptosis in NP cells is accompanied by inflammation. Hence, inhibiting the excessive apoptosis of NP cells may be a potential way to alleviate IDD. It was not initially clear whether CXCR4 inhibition had the capacity to suppress cell apoptosis. Thus, caspase 3 and caspase 8, which are two key factors in the regulation of apoptosis, were detected. Earlier studies have demonstrated that caspase 3 and caspase 8 participate in apoptosis by regulating oxidative stress and inflammatory response. The findings of this study also lend strong support for the view that caspase 3 and caspase 8 are activated by the increased expression of CXCR4. Therefore, AMD 3100 is a super inhibitor that

suppresses the influence of CXCR4, leading to reduction of the apoptosis of NP cells apoptosis in IDD.

CONCLUSION

The findings of the present study reveal that CXCR4 inhibition may be a useful approach to treat intervertebral disc diseases. Future studies are required to further explore potent drugs that would target CXCR4 for the treatment of IDD *in vivo*.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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