Original Article
Inhibition of intervertebral degenerative disc disease by regulating NF-κB

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Abstract: The incidence of intervertebral degenerative disc disease (IDDD) is gradually increasing with change of life style, thus causing severe economic burden for patients. With unfavorable prognosis, the pathogenesis mechanism of IDDD is still unclear yet. Current opinions agreed that inflammatory factor is the critical factor for IDDD occurrence as it can degrade matrix of intervertebral disc. As one negative immune regulator, the function and mechanism of interleukin-10 (IL-10) in IDDD are still unknown yet. Nuclei pulposus of intervertebral disc were collected from IDDD patients to separate and culture disc nucleus *in vitro*. Cells were treated with different concentrations (100 mM, 50 mM and 20 mM) of IL-10. MTT assay was employed to test the effect on cell proliferation, while caspase 3 activity was measured by test kit. Expression of ADAMTS-4 gene was measured by real time PCR. NF-κB expression was examined by real time PCR and Western blot. Levels of inflammatory factor IL-1 and IL-6 were analyzed by ELISA. IL-10 functions on nucleus pulposus of IDDD patients to facilitate cell proliferation, inhibit caspase 3 activity, decrease ADAMTS-4 mRNA expression, and inhibit secretion of inflammatory factor IL-1 and IL-6 ($P<0.05$ compared to control group). With higher IL-10 concentration, more potent effects occurred ($P<0.05$ comparing between high dosage and low dosage groups). IL-10 inhibits ADAMTS-4 expression and inflammation via mediating NF-κB, thus participating in regulation of extracellular matrix metabolism, inhibiting nuclei pulposus apoptosis, facilitating cell proliferation, and thus alleviating IDDD.

Keywords: IL-10, intervertebral disc degenerative disease, NF-κB, nuclei pulposus, inflammation

Introduction

With life style transition, change with life habitat, population aging, less exercise and more sedentary working, the incidence of intervertebral disc degenerative disease (IDDD) is gradually increasing in clinics [1, 2]. Frequency of IDDD in China is increasing by years with aging of patient population [3]. IDD can cause low back pain, which is one common complication that may cause the inability to work or even morbidity, leading to severe pain for patients and heavy social burdens [4, 5]. Although low back pain can be caused by various reasons including IDDD, lumbar disc herniation, lumbar spondylolisthesis, or lumbar spinal canal stenosis, IDDD is the major reason causing low back pain, making it one major public health issue worldwide [6, 7].

In pathogenesis of IDDD, extracellular matrix (ECM) of intervertebral disc undergoes molecular and mechanical changes under combined effects of various physical and chemical factors, blurring the boundary between fibrous ring and nuclei pulposus [8]. Due to the change of collagen fiber composition of intervertebral disc ECM, disrupted overall structure, plus decrease or loss of proteoglycan, which is one early marker of early phase of IDDD, eventually leading to dehydration of nuclei pulposus and decreasing of intervertebral disc load [9, 10]. Nuclei pulposus is one type of chondrocytic cells, and occupies about half area of the whole intervertebral disc [11]. Lower number and structural damage of nuclei pulposus decrease tissue elasticity, disrupting biophysical function of intervertebral disc [12]. To date, the pathogenesis of IDDD is still unclear. Inflammatory factor has been

known to participate in ECM metabolism and cell proliferation, and is thus recognized as critical factor for IDDD pathogenesis to induce ECM degradation of intervertebral disc and nuclei pulpous injury [13, 14]. Interleukin-10 (IL-10) is one pluripotent negative regulator produced by Th2 cells, activated B cells, monocytes and macrophage, and participates in biological modulation of immune cells, inflammatory cells and tumor cells, thus playing an important role in various diseases including autoimmune disease, severe infection, tumor and transplantation immunity [15, 16]. As one negatively immune regulator, the function and mechanism of IL-10 in IDDD is still unclear.

Materials and methods

General information

Five IDD patients (3 males and 2 females, aging between 29 and 45 years, average age = 37.2±3.6 years) who were diagnosed as IDDD and received surgery in the First Affiliated Hospital of Baotou Medical college from January 2015 to January 2016 were recruited.

Inclusive criteria: IDDD was diagnosed by lumbar MRI. All patients were degenerative stage III according to Christian MRI standard. Patient ages were all younger than 45 years. Patients received surgery for removal of nucleus pulpous or intervertebral disc.

Exclusive criteria: Those patients with other lumbar disc disease, infectious disease, malignant tumor, severe diabetes, liver/kidney disease, pulmonary fibrosis, bone metabolism disorder, systemic immune disease or tumor complications. Sample collection of human lumbar intervertebral disc nucleus pulpous has obtained informed consent from patients and families. This study has been approved by the ethical committee of the First Affiliated Hospital of Baotou Medical college.

Reagent

Type II collagenase, Trizol reagent and IL-10 were all purchased from Sigma (US). RNA extraction kit, RT-PCR primer, reverse transcription (RT) kit and real-time PCR kit were all purchased from Axygen (US). PVDF membrane was obtained from Pall Life Sciences (US). Western blot reagent was purchased from Beyotime

(China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human NF-κB monoclonal anibody and goat anti-rabbit IgG with horseradish peroxidase (HRP) label were purchased from Cell signaling (US). DMEM/F12 medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone (US). DMSO and MTT powder were purchased from Gibco (US). Trypsin digestion buffer was purchased from Sigma (US). ELISA kit for IL-1 and IL-6 was purchased from R&D (US). Surgical microscope was purchased from Suzhou Instrument (China). Caspase 3 activity assay kit was purchased from Cell Signaling (US). ABI7900 HT real-time PCR cycler was purchased from ABI (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US).

Separation, culture and grouping of primary nucleus pulpous

Nucleus pulpous or intervertebral disc tissues removed during the surgery were rinsed by sterile saline repeated. In sterilized culture dish, fibrous ring around intervertebral disc and other non-nucleus pulpous mesenchymal tissues were removed. Samples were processed in sterilized ultrapure work station, and were rinsed by D-Hank solution to completely remove blood inside nucleus pulpous tissues. Samples were cut into 1 mm^3 size cubes, and were digested in 0.1% type II collagenase at 37°C incubator for 45 min. The supernatant was saved and centrifuged at 1500 rpm for 5 min, and was transferred to 50 ml culture flask, which contained 4 ml fresh DMEM medium. After incubation in a humidified incubator for 24~48 h at 37°C with 5% CO_2, LECs were inoculated into 6-well plate at 1×10^5 density, using 90% high glucose DMEM/F12 medium containing 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were cultured in a 37°C with 5% CO_2, with medium changing every 3 days. Cells were passed every three days until reaching 80%~90% confluence. When passing cells, old medium was removed, cells were rinsed in D-Hanks solution, digested using 0.25% trypsin and 0.02% EDTA for 5~10 min, and were passed at 1:2 ratio. 2nd to 5th generation of log-phase cells were divided into 4 groups: control group and high (100 mM), moderate (50 mM) and low (20 mM) concentration of IL-10. After 48 h treatment, further experiment was performed.

Table 1. Primer sequence

Target gene	Forward gene 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
ADAMTS-4	AGGTCTACTCGCCACACAGT	GGGTCAGCCTTAAGCTTATT
NF-κB	CAGGTCTTGGATCCACACGCC	TCATTAGCCATAGCGTCTAACC

MTT assay for the effect of IL-10 on cell proliferation

Nucleus pulpous cells at log-phase were digested, counted and seeded into 96-well plate at 3000 cells per well containing DMEM/F12 medium with 10% FBS. Cells were then randomly divided into high, moderate and low IL-10 groups as abovementioned. After 48-hour incubation, 20 µl sterile MTT solution was then added into each test well in triplicates. With 4 h continuous culture, the supernatant was completely removed, with the addition of 150 µl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group. Each experiment was repeated in triplicates for statistical analysis.

ELISA for IL-1 and IL-6 expression level in all cells

Expression levels of IL-1 and IL-6 in cell culture supernatant were quantified by ELISA following the manual instruction of test kits. In brief, 96-well plate was added with 50 µl serially diluted samples, which were used to plot standard curves. 50 µl test samples were then added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 sec vortex. The rinsing procedure was repeated for 5 times. 50 µl enzyme labelling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 µl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 µl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective OD values. Sample concentration was further deduced based on OD values and regression function.

Caspase 3 activity assay

Caspase 3 activity in cells was measured following manual instruction of test kit. In brief, cells were digested in trypsin, and were centrifuged at 600 g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Absorbance (A) values at 450 nm wavelength was measured to reflect caspase 3 activity.

Real time PCR for ADAMTS-4 and NF-κB mRNA expression

mRNA was extracted from all cells using Trizol reagents. cDNA was synthesized reverse transcription following manual instruction. Primer 6.0 was used to design PCR specific primer (**Table 1**), which was synthesized by Invitrogen (China). Real-time PCR was used to test target gene expression under the following conditions: 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Fluorescent quantitative PCR was used to collect data. CT values of standard samples were calculated based on internal reference gene GAPDH for plotting standard curve. Semi-quantitative analysis was performed by $2^{-\Delta Ct}$ method.

Western blot for NF-κB protein expression

Total proteins were extracted from all cells. In brief, tissues were mixed with lysis buffer for 15~30 min iced incubation. Using ultrasonic rupture (5 s, 4 times) and centrifugation (10000 g, 15 min), proteins were quantified from the supernatant and were kept at -20°C for Western blotting. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method. Non-specific binding sites were blocked by 5% defatted milk powders for 2 hours. Anti-NF-κB monoclonal antibody (1:1000) or anti-β-actin monoclonal antibody (1:2000) was applied for 4°C overnight incubation. Goat anti-rabbit IgG (1:2000) was then added for 30-min incubation. After PBST washing and ECL development for 1 min, the mem-
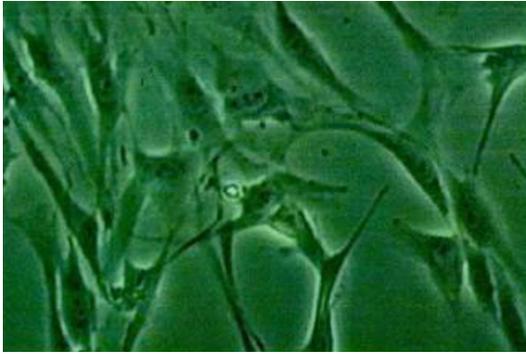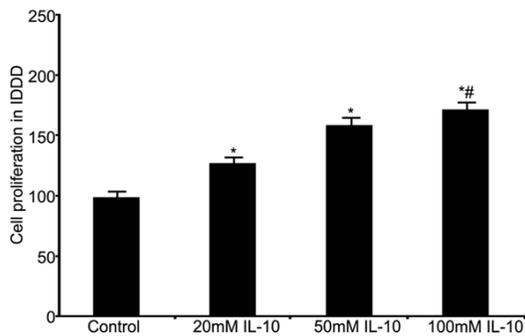
Figure 1. Nucleus pulpous morphology at 2nd generation (×200).

Figure 2. Effect of IL-10 on proliferation of nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.

brane was exposed under X-ray. An imaging analyzing system and Quantity one software were then used to scan X-ray films and to detect the density of bands with repeated measures (N = 4).

Statistical analysis

SPSS 19.0 software was used to collect all data, of which measurement data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. A statistical significance was defined when P<0.05.

Results

Morphology of nucleus pulposus

Under bright field microscope, primary culture cells showed polygon shape with short synapse formation. With extended culture time, cells were further elongated plus longer synapse and color change of nucleus (**Figure 1**).
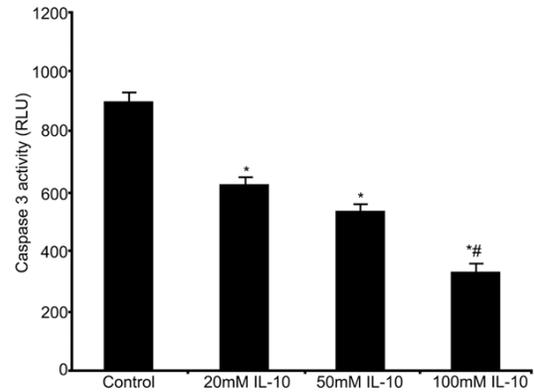

Figure 3. Effect of IL-10 on caspase 3 activity of nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.

Effect of IL-10 on nucleus pulpous proliferation

MTT assay was used to test the effect of different concentrations of IL-10 on proliferation of nucleus pulpous. Results showed facilitated proliferation in IDDD samples after IL-10 (P< 0.05 compared to control group). With elevated IL-10 dosage, the facilitating effect on proliferation became more potent (**Figure 2**).

Effects of IL-10 on caspase 3 activity of nucleus pulpous

Caspase 3 activity assay kit was used to detect the effect of different concentrations of IL-10 on caspase 3 activity of IDDD nucleus pulpous. Results showed that IL-10 effectively inhibited caspase 3 activity in nucleus pulpous (P<0.05 compared to control group). With elevated dosage, the inhibitory effect on caspase 3 activity became more potent (**Figure 3**). These results suggested that IL-10 could facilitate proliferation of IDDD nucleus pulpous via suppressing apoptosis, thus inhibiting IDDD condition.

Effects of IL-10 on inflammatory factor expression in nucleus pulpous

ELISA was used to detect the effect of different concentrations of IL-10 on inflammatory factors IL-1 and IL-6 expressions in the supernatant of cultured nucleus pulpous of IDDD. Results showed that IL-10 significantly inhibited the expression of inflammatory factors IL-1 and IL-6 in nucleus pulpous (P<0.05 compared to control group). With elevated dosage, the inhibitory effect on IL-1 and IL-6 became more potent

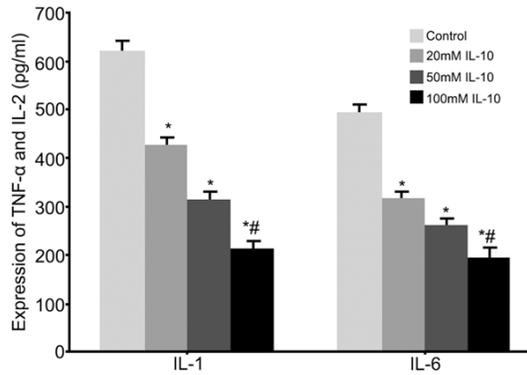**Figure 4.** Effects of IL-10 on expression of inflammatory factor IL-1 and IL-6 in nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.
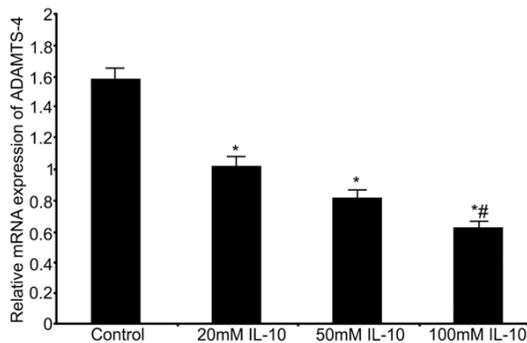

Figure 6. Effects of IL-10 on NF-κB mRNA expression in nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.

Figure 5. Effect of IL-10 on ADAMTS-4 mRNA level in nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.

Figure 7. Effects of IL-10 on NF-κB protein expression in nucleus pulpous. A: NF-κB protein expression in nucleus pulpous after IL-10 treatment. B: Analysis of the effect of IL-10 on NF-κB protein expression in nucleus pulpous. *, P<0.05 compared to control group; #, P<0.05 compared to 20 mM IL-10 group.

(**Figure 4**). These results suggested that IL-10 could inhibit secretin of inflammatory factors by IDDD nucleus pulpous, thus modulating IDDD progression.

Effects of IL-10 on ADAMTS-4 mRNA in nucleus pulpous

Real time PCR was used to detect the effect of different concentrations of IL-10 on ADAMTS-4 mRNA level in the supernatant of cultured nucleus pulpous of IDDD. Results showed that IL-10 significantly inhibited the expression of ADAMTS-4 mRNA expression in nucleus pulpous (P<0.05 compared to control group). With elevated dosage, the inhibitory effect on ADAMTS-4 became more potent (**Figure 5**).

Effects of IL-10 on NF-κB expression in nucleus pulpous

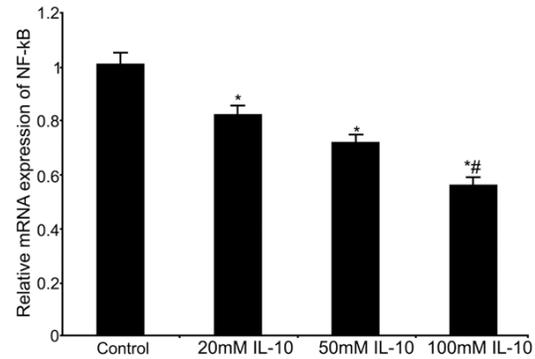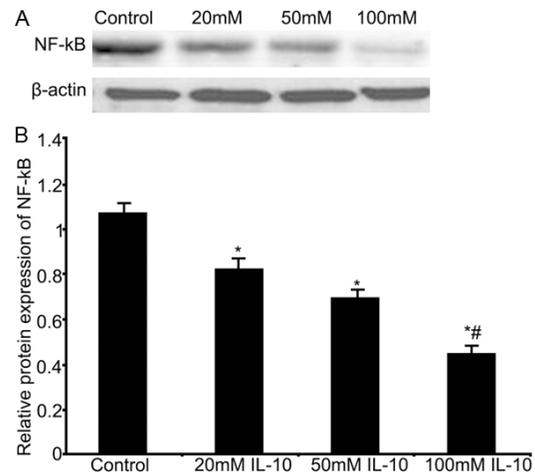Real time PCR and Western blot were used to detect the effect of different concentrations of

IL-10 on NF-κB mRNA and protein expression in the nucleus pulpous of IDDD, respectively. Results showed that IL-10 significantly inhibited mRNA and protein expression of NF-κB in nucleus pulpous (P<0.05 compared to control group). With elevated dosage, the inhibitory effect on NF-κB became more potent (**Figures 6, 7**).

Discussion

Various factors including genetics, body aging, decreased immune escape potency in nucleus pulpous region, apoptosis or death of nucleus pulpous underlie IDDD pathogenesis [17, 18]. Nucleus pulpous of intervertebral disc contains proteoglycan and type II collagen, to maintain

normal structure of intervertebral disc, and to exert certain functions on metabolism and biophysical property of intervertebral disc. The GAG structure contains abundant water to maintain the elasticity of intervertebral disc nucleus pulpous to guarantee durance of intervertebral disc [19, 20]. Therefore during IDDD pathogenesis, the loss of GAG and further dehydration lead to structural and functional change of nucleus pulpous, leading to IDDD occurrence [21]. Under normal physiological conditions, the synthesis and degradation of GAG stays at a homeostatic status to maintain normal function of intervertebral disc. Under the interruption of exogenous factors, the expression of degrading enzyme was increased, causing over-degradation of ECM, thus interrupting dynamic balance of body GAG, leading to IDDD [22].

Recent studies found the specificity of AD-AMTS-4, which belongs to aggregated proteoglycan enzyme ADAMTS family, as its showed unique degrading activity for aggregated proteoglycan but not for other ECM components such as collagen or fibronectin [23]. The elevated secretion of inflammatory factor further causes inflammation and injury of nucleus pulpous [13, 14]. Therefore, the identification of factors to inhibit inflammatory factors could inhibit GAG degradation via modulating ADAMTS-4, further decreasing apoptosis of nucleus pulpous or death. As one anti-inflammatory factor, IL-10 exerts its function for suppressing inflammatory response and antagonizing inflammatory mediator [24]. This study demonstrated that IL-10 could facilitate proliferation of nucleus pulpous, inhibit caspase 3 activity, suppress ADAMTS-4 expression, and inhibit secretion of inflammatory factors IL-1 and IL-6 in a dose-dependent manner. These results showed that as one novel regulatory factor, IL-10 might inhibit progression of IDDD. Further studies on its mechanism found that the activation of NF-κB could aggravate recruitment and adhesion of neutrophil and macrophage, to release abundant free oxygen radicals, facilitate secretion of inflammatory factors, and aggravate inflammation, leading to the destruction of integrity of intervertebral disc tissues and nucleus pulpous, thus aggravating edema of intervertebral disc and progression of IDDD [25]. In summary, this study found that IL-10 could inhibit IDDD progression via inhibiting gene and protein expression of NF-κB.

Conclusion

IL-10 could inhibit ADAMTS-4 and inflammation via regulating NF-κB, thus participating regulation of ECM metabolism, inhibiting apoptosis and facilitating proliferation of nucleus pulpous, thus suppressing IDDD condition.

Disclosure of conflict of interest

None.

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