Original Article
Inhibition of osteoarthritis chondrocyte proliferation by IL-10 via modulating NF-κB and related mechanisms

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Abstract: Osteoarthritis (OA) is one common disease in clinics and causes joint pains for patients. Most drugs treating OA can only relieve symptoms due to the complicated pathogenesis mechanism, with inflammatory factor as one major reason. Interleukin-10 (IL-10) is one negative immune regulatory factor, but with unclear mechanism in OA. Joint cartilage tissues were collected from both OA patients and normal people for measuring IL-10 expression by real-time PCR. OA chondrocytes were separated, cultured and assigned into control, 10 μM and 20 μM IL-10 treatment group. Cell proliferation and apoptosis were measured by MTT and flow cytometry, respectively. Caspase 3 activity was measured by test kit. NF-κB expression was measured by Western blot, and levels of tumor necrosis factor (TNF)-α and IL-6 were tested by enzyme linked immunosorbent assay (ELISA). OA patients had decreased IL-10 expression (P<0.05 compared to control group). IL-10 treatment suppressed proliferation of OA chondrocytes, facilitated cell apoptosis, enhanced Caspase 3 activity, decreased NF-κB, TNF-α or IL-6 expression (P<0.05 compared to control group). With increased dosage, more potent effects followed (P<0.05). IL-10 is down-regulated in OA. The over-expression of IL-10 can suppress cell proliferation or NF-κB expression, decrease secretion of inflammatory factors, thus retarding OA progression.

Keywords: Osteoarthritis, IL-10, NF-κB, inflammation, cell proliferation, apoptosis

Introduction

Osteoarthritis (OA) is one common disease in orthopedics. As one chronic degenerative joint lesion, OA is frequently occurred in elder patients, leading to limb motility, thus severely affecting patient's life quality and brining heavy burdens for the public health [1, 2]. Previous studies showed that multiple inducing factors such as elder age, higher body mass index (BMI), trauma, labor hurt, joint deformation, inherited joint abnormality and genetics all contribute to OA [3, 4]. OA is frequently occurred secondary to joint cartilage degeneration or bone hyperplasia, and is thus also named as bone joint disease, degenerative arthritis, elder arthritis, or hyperplasia arthritis [5, 6]. Major pathological features of OA include joint cartilage degeneration or injury, reactive hyperplasia in joint edge or subchondral bones [7].

Clinical symptoms of OA include chronically developed joint pains in the morning time, and relived pains after activity, plus swelling joint tissues, compressed joint pains, joint stiffness, inhibited motility and joint deformation [8]. OA has complicated pathogenesis mechanism, which has not been fully illustrated.

Immune and inflammatory response is one critical factor for OA pathogenesis [9, 10]. IL-10 is one pluripotent negative regulatory, and is mainly produced by Th2 cells, activated B cells, monocytes, and macrophage [11, 12]. It is involved in biological regulation of immune cells, inflammatory cells and tumor cells, thus exerting important roles in autoimmune disease, severe infection, tumor and transplant immunity [13, 14]. The role of IL-10 as one negative immune modulator in OA has not been illustrated.

Table 1. Primer sequences

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
IL-10	CAGCTCCTACCAGCTTAAG	GCACGATTGACTGCTTTGTGA

Materials and methods

General information

A total of 15 OA patients who were admitted in the Second Affiliated Hospital of Henan University of Chinese Medicine from December 2015 to June 2016 were recruited in this study, including 8 males and 7 females, aging between 49 and 62 years (average age = 55±8 years). All patients received joint replacement surgery. Exclusion criteria includes those patients with history of surgery, chemotherapy or radiotherapy; complicated with other diseases such of infectious disease, malignant tumor, severe liver/kidney disease, pulmonary fibrosis, bone metabolic disorder, secondary kidney hypertension, systemic immune disorder or cancer complications. Those patients who were not willing to cooperate with this study or follow-ups were also excluded. A cohort of 15 patients with knee joint fracture were recruited as the control group, which consisted of 9 males and 6 females, aging between 48 and 68 years (average age = 54±9 years). General information had no significant difference between two groups. All collected samples were placed in liquid nitrogen. This study had been approved by the ethical committee of the Second Affiliated Hospital of Henan University of Chinese Medicine. All participants have signed informed consents of this study.

Major reagents and equipment

Trizol reagent and type II collagenase were purchased from Sigma (US). RNA extraction kit, RT-PCR primers, reverse transcription (RT) kit, real-time PCR reagent were purchased from Axygen (US). PVDF membrane was purchased from Pall Life Science. Western blot reagents were purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human NF-κB and goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell signaling (US). DMEM/F12 culture medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powder were purchased from R&D (US). Surgical microscopic instrument was purchased from Suzhou Medical Instrument (China). Annexin V-PI kit was purchased from BD (US). Caspase 3 activity assay kit was purchased from Cell signaling (US). FACSCalibur flow cytometry was purchased from BD (US). ABI7900 HT real-time PCR was purchased from ABI (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US).

Real-time PCR for IL-10 expression in OA cartilage tissues

Tissue cubes were homogenized under liquid nitrogen. After mixing in lysis buffer, Trizol reagent was used to extract tissue proteins. DNA reverse transcription was performed following the instruction of test kit (Axygen, US). Primers were designed based on target gene sequence using PrimerPremier 6.0, and were synthesized by Invitrogen (China) as **Table 1**. Real-time PCR was performed for measuring target gene expression under following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Data were collected and calculated for CT values of all standards and samples based on fluorescent quantification and using GAPDH as the internal reference. Standard curve was plotted for semi-quantitative analysis by $2^{-\Delta\Delta Ct}$ method.

Separation, culture and grouping of primary osteoarthritis chondrocytes

OA cartilage tissues were collected during surgery. With repeated rinsing in saline, connective tissues around bone joint cartilage was removed in sterile dish. Each sample was processed in sterile workstation, with rinsing in D-Hank solution until removing all blood contamination in bone joint cartilage. Tissues were cut into cubes with 1 mm³ size and digested in 0.1% type II collagenase for 37°C shaker incubation for 45 min. The supernatant was removed and the mixture was centrifuged at 1500 rpm for 5 min, and were removed into 50 ml culture flask containing 4 ml fresh DMEM medium. Cells were cultured at 37°C with 5% CO_2 in a humidified chamber for 24-48 h cul-

ture. LECs cells were seeded into 6-well plate at 1×10^5 cells per cm² density, with 90% high glucose DMEM/F12 medium (containing 100 U/mL penicillin, 100 μg/ml streptomycin) and 10% FBS, and were cultured at 37°C with 5% CO_2. Culture medium was changed every 3 days. Cells were passaged when paving 80% to 90% of dish bottom. During 1:2 passage, cells were rinsed in D-Hanks solution, and digested for 5-10 min using trypsin-0.02% EDTA buffer. Cells at log-growth phase with the 2nd-5th generation were selected for experiments, and randomly assigned into three groups: control group, 10 μM IL-10 treatment group, and 20 μM IL-10 treatment group. All cells were cultured for 48 h for further experiments.

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

OA chondrocytes at log-growth phase were inoculated into 96-well plate containing DMEM/F12 medium with 10% FBS at 5×10^3 cells. After 24 h culture, the supernatant was discarded. Cells were randomly assigned into control group, 10 μM IL-10 treatment group, and 20 μM IL-10 treatment group as abovementioned. After 48 h treatment, 20 μL sterile MTT as added into each well in triplicates. After 4 h incubation, the supernatant was discarded, followed by the addition of 150 μl DMSO per well for 10 min shaker incubation until complete resolving of crystal violet. A microplate reader was used to measure absorbance (A) values at 570 nm of each well for calculating proliferation rate. Each experiment was repeated for more than three times.

ELISA for measuring TNF-α and IL-6 in supernatant

All samples were measured for TNF-α and IL-6 expression in supernatant by ELISA kit, following manual instruction of test kit. 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 sequen-

tially 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 A values. Sample concentration was further deduced based on optical density (OD) values using the regression function.

Caspase 3 activity assay

Caspase 3 activity in cells was evaluated from all groups following manual instruction of test kits. In brief, cells were digested by 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. Optical density (OD) values at 405 nm wavelength was measured to calculate caspase 3 activity.

Flow cytometry for effects of IL-10 on cell apoptosis

Cells from all groups were digested, counted and inoculated into 50 mL flask at 5×10^5/mL and were randomly divided into three groups as described earlier in triplicates. Cells were digested and collected at 2×10^6 and were washed in 1×PBS at 1000 rpm for 5 min. Cells were fixed in 75% pre-cold ethanol, and were incubated at 4°C overnight. Ethanol was discarded, with washing in 1×PBS at 1000 rpm for 5 min centrifugation. Cells were then re-suspended in 0.8 mL 1×PBS containing 1% BSA, and was mixed with 100 μg/mL PI staining dye (3.8% sodium citrate, pH 7.0), and 10 μL RNAaseA (10 mg/mL) for 37°C dark incubation for 30 min. FCSExpress 3.0 software was used to analyze all collected data from flow cytometry instrument.

Western blot for NF-κB protein expression

Cell proteins were firstly extracted. In brief, lysis buffer was used to lyse cells on ice for 15-30 min, followed by ultrasound rupture (5 s ×4) and centrifugation (4°C, 10000 g, 15 min).
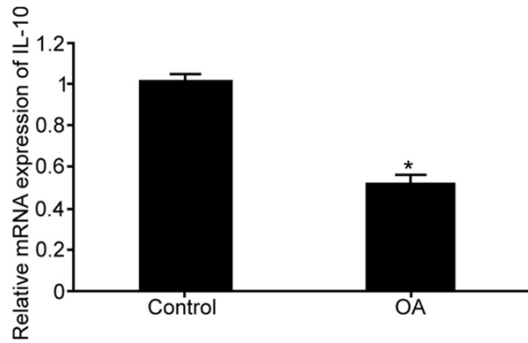
Figure 1. IL-10 expression in OA cartilage tissues. *, P<0.05 compared to control group.

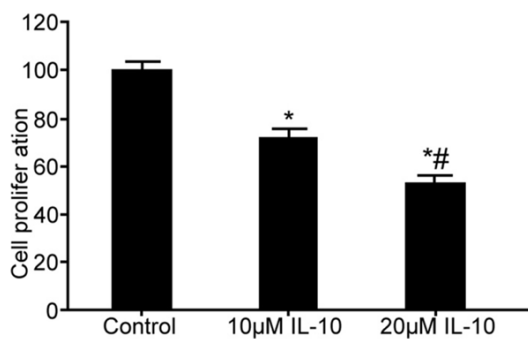**Figure 2.** Effects of IL-10 on proliferation of OA chondrocytes. *, P<0.05 compared to control group; #, P<0.05 compared to 10 µM IL-10 group.
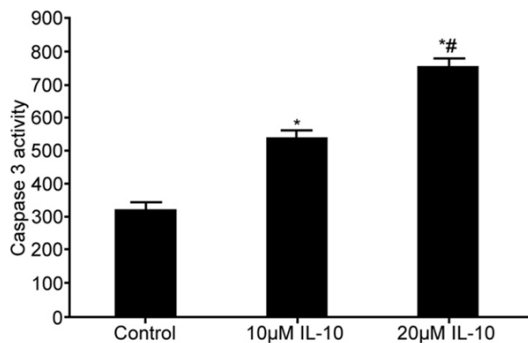

Figure 3. Effects of IL-10 on Caspase 3 activity in OA chondrocytes. *, P<0.05 compared to control group; #, P<0.05 compared to 10 µM IL-10 group.

Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated using 10% SDS-PAGE gel, and were transferred to PVDF membrane using semi-dry method (100 mA, 1.5 h). Non-specific background was removed by 5% defatted milk pow-

der at room temperature for 2 h, followed by the addition of, anti-NF-κB monoclonal antibody (1:1000) or anti-β-actin monoclonal antibody (1:2000) in 4°C overnight incubation. On the next day, the membrane was rinsed in PBST, and incubated with secondary antibody for 30 min dark incubation. After PBST rinsing, ECL reagent was used to develop the membrane for 1 min, followed by exposure under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N=4) for further analysis.

Statistical processing

SPSS19.0 was used for analysis. Measurement data were presented as mean ± standard deviation (SD). Comparison of means among multiple groups was performed by one-way analysis of variance (ANOVA). A statistical significance was defined when P<0.05.

Results

Expression of IL-10 in OA cartilage tissues

Real-time PCR was used to test IL-10 expression in OA cartilage tissues. Results showed significantly lower IL-10 mRNA expression in OA cartilage tissues (P<0.05 compared to control group, **Figure 1**).

Effects of IL-10 on OA chondrocyte proliferation

MTT assay was used to test the effect of different concentrations of IL-10 on proliferation of OA chondrocytes. Results showed that IL-10 treatment inhibited chondrocytes proliferation (P<0.05 compared to control group). With increased dosage of IL-10, its facilitating effects on proliferation became more potent (**Figure 2**).

Effects of IL-10 on caspase 3 activity in OA chondrocytes

Assay kit was used to measure the effect of different concentrations of IL-10 on Caspase-3 activity of OA chondrocytes. Results showed that treatment on OA chondrocytes using IL-10 significantly facilitated Caspase 3 activity in OA chondrocytes (P<0.05 compared to control
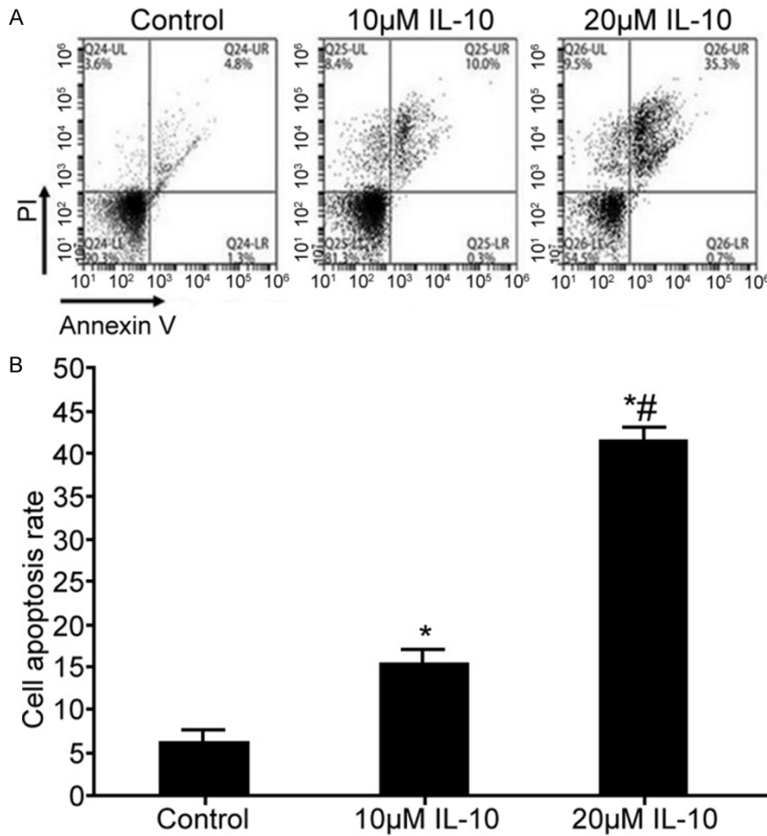
Figure 4. Effects of IL-10 on apoptotic activity of OA chondrocytes. A. Flow cytometry analyzed apoptotic activity of OA chondrocytes; B. Analysis of apoptotic rate of OA chondrocytes. *, P<0.05 compared to control group; #, P<0.05 compared to 10 µM IL-10 group.
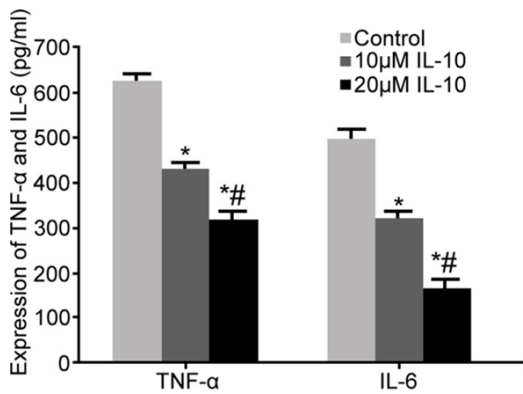

Figure 5. Effects of IL-10 on inflammatory factor expression in OA chondrocytes. *, P<0.05 compared to control group; #, P<0.05 compared to 10 µM IL-10 group.

Effects of IL-10 on apoptotic activity of OA chondrocytes

Flow cytometry was used to measure the effect of different concentrations of IL-10 on apoptotic activity of OA chondrocytes. Results showed that IL-10 treatment on OA chondrocytes significantly facilitated cell apoptosis (P<0.05 compared to control group). With increased dosage, the facilitating role on cell apoptosis became more potent (**Figure 4**). These results showed that IL-10 could inhibit OA chondrocyte proliferation via facilitating OA chondrocyte apoptosis, thus modulating OA chondrocytes.

Effects of IL-10 on inflammatory factors in OA chondrocytes

ELISA was used to measure the effects of different concentrations of IL-10 on inflammatory factors TNF-α and IL-6 levels in supernatant of cultured OA chondrocytes. Results showed that IL-10 treatment on OA chondrocytes significantly suppressed their expression of inflammatory factors TNF-α and IL-6 (P<0.05 compared to control group). With increased dosage, the inhibitory effects of IL-10 on the expression of inflammatory factors TNF-α or IL-6 became more potent (**Figure 5**). These results showed that IL-10 could suppress the secretion of inflammatory factors from OA chondrocytes, thus modulating OA progression.

Effects of IL-10 on NF-κB expression in OA chondrocytes

Western blot was used to measure the effects of different concentrations of IL-10 on protein expression of NF-κB in OA chondrocytes. Results showed that IL-10 significantly inhibited NF-κB expression in OA chondrocytes (P<0.05 compared to control group). With increased
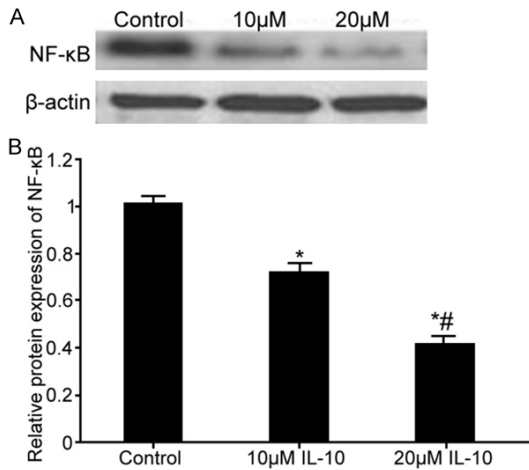
group). With increased dosage, its facilitating functions on Caspase 3 activity become more potent (**Figure 3**).

Figure 6. Effects of IL-10 on NF-κB expression in OA chondrocytes. A. NF-κB protein expression in OA chondrocytes after IL-10 treatment; B. Analysis of NF-κB protein expression in OA chondrocytes by IL-10 modulation. *, P<0.05 compared to control group; #, P<0.05 compared to 10 μM IL-10 group.

dosage, the inhibitory effect of IL-10 on NF-κB protein expression become more potent (**Figure 6**).

Discussion

As one common disease in orthopedics, OA compromises joint activity and causes joint deformation. The pathogenesis mechanism of OA has not been completely illustrated. During OA progression, undamaged joint cartilage tissues showed active proliferation of chondrocytes with active metabolism to secrete large amounts of proteoglycan and collagen, leading to the destruction of extracellular matrix (ECM) [15]. With development of OA, subchondral bones detaches from coverage region by calcified cartilage, forming osteophyte to bridge joint cavity [16]. Therefore, OA mainly leads to joint cartilage cell proliferation, differentiation, ECM degradation, and progressive loss of joint cartilage component, forming joint osteophyte [17, 18]. Therefore, OA chondrocytes play an important role in disease progression. Increased secretion of inflammatory factors causes inflammation to induce joint injury [19]. Therefore, the identification of factors for facilitating OA chondrocyte apoptosis and inhibiting cell proliferation can effectively impede joint injury.

This study demonstrated decreased IL-10 expression in OA tissues, whilst IL-10 treatment could suppress chondrocyte proliferation of OA tissues, thus facilitating Caspase 3 activity, inducing chondrocyte apoptosis, and inhibiting secretion of inflammatory factors TNF-α or IL-6. IL-10 can suppress OA progression via inhibiting NF-κB protein expression. Activation of NF-κB can aggravate enrichment and adhesion of neutrophils and macrophage, releasing large amounts of free oxygen radicals to facilitate secretion of inflammatory factors and to aggravate inflammatory response, thus destructing integrity of OA tissues, causing chondrocyte proliferation, aggravating joint injury, eventually leading to OA progression [20, 21]. In Caspase family, Caspase 3 is the most potent member, and its enhanced activity leads to irreversible cell damage [22, 23]. IL-10 is one anti-inflammatory factor and can antagonize inflammatory mediator to suppress inflammation [24]. Currently, the role or mechanism of IL-10 in OA has not been reported yet. Our results showed that IL-10 as one novel regulatory factor, could mediate OA progression. Further studies are required to investigate possible mechanism.

IL-10 has decreased expression in OA tissues. The enhancement of IL-10 expression can inhibit cell proliferation, suppress NF-κ expression, and decrease inflammatory factor secretion via facilitating OA chondrocyte apoptosis, thus retarding OA progression.

Disclosure of conflict of interest

None.

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