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Original Article

IL-6 mediates miR-200 to influence chondrocytic proliferation in osteoarthritis

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Abstract: Osteoarthritis (OA) is a degenerative joint disease that seriously impairs human health. The main pathological changes are articular cartilage degeneration, joints marginal bone hyperplasia. Advanced OA can cause joint deformities and dysfunction. Pervious research found that IL-6 expression elevated in OA patients, while the role of IL-6 in OA is still unclear. This study aimed to investigate IL-6 mechanism by observing its impact on OA chondrocytes proliferation. 15 cases of cartilage samples were collected from OA patients receiving total joint arthroplasty in XXX hospital. OA cartilage primary cells were treated by type II collagenase and seeded in 96-well plate. CCK8 assay was applied to detect cell proliferation after IL-6 treatment. Real time PCR was used to test miR-200 expression. MiR-200 impact on cartilage proliferation was observed by overexpression and inhibition. Primary chondrocytes grew well after 72 h obtained by type II collagenase. CCK8 assay showed that IL-6 treatment weakened chondrocyte proliferative ability. Real time PCR revealed that miR-200 level decreased to 0.63 times in IL-6 group. Compared with negative control, miR-200 mimic transfection enhanced chondrocyte proliferation, while miR-200 inhibitor transfection weakened chondrocyte proliferative ability. IL-6 inhibits miR-200 expression in OA chondrocytes. IL-6 suppresses chondrocyte proliferation by downregulating miR-200 level.

Keywords: IL-6, miR-200, osteoarthritis, proliferation

Introduction

Osteoarthritis (OA) is a type of chronic degenerative joint disease that is commonly seen in middle aged and elderly people [1]. OA often involves multiple joints and mainly manifests as joint swelling, pain, deformity, and limited activities [2]. OA is caused by various factors combined action though its pathogenesis is still not fully elucidated. It was reported that OA occurrence was related to articular cartilage damage, inflammatory cells infiltration, fibrosis, and extracellular matrix degradation [3]. Articular cartilage is composed of chondrocytes and cartilage matrix, while articular cartilage degeneration is mainly presented as cartilage matrix synthesis and catabolism imbalance [4]. Chondrocytes are the main source of cartilage matrix synthesis, which is of great significance for maintaining cartilage matrix steady state [5]. Though the balance mechanism is still unclear, IL-6 has been verified to play a critical role to maintain such balance [6]. IL-6 belongs to interleukin that is synthetized and secreted by macrophages, chondrocytes, and osteoclasts [7]. It was found that IL-6 played an important role in mediating inflammatory signaling pathways, chondrocyte proliferation, angiogenesis, and bone metabolism [8]. Previous studies about IL-6 and OA mainly focused on IL-6 level changes in OA patients, but were not in-depth molecular biology research. The development of miRNA investigation provides new thought for the mechanism of IL-6 effect in OA. MiRNA is a class of small non-coding RNA with high fidelity. Mature miRNA inhibits gene translation process to regulate gene expression by targeting mRNA 3' UTR [9]. Research showed that miRNAs accounted for 1% regulated nearly 30% of gene expression and participated in a variety of biological processes, such as cell activation, proliferation,

Table 1. Primer sequence

Gene	Forward	Reverse
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
miR-200	UUAAUCCUUCCAAGAUGUCUUA	CAGUACUUUUGUGUAGUACAA

differentiation, apoptosis, and canceration, etc. [10]. Previous research showed miR-200 low expressed in OA patients [11], suggesting that miR-200 abnormal expression was related to OA occurrence. This study intended to investigate IL-6 mechanism by observing its impact on OA chondrocytes proliferation.

Materials and methods

Main reagents and instruments

DMEM medium (Gibco, USA); MEM medium (Gibco, USA); Fetal bovine serum FBS (Gibco, USA); Trypsin (Gibco, USA); type II collagenase (Invitrogen, USA); Lipofectamine 2000 (Invitrogen, USA); Total RNA extraction reagent Trizol (Invitrogen, USA); RT-PCR kits (Toyobo Life Science, Japan); SYBR fluorescent dyes (Toyobo Life Science, Japan); CCK8 kit (Dojindo, Japan); Enzyme-linked immune detector (Bio-tek, USA); Dual luciferase reporter detection system (Promega, USA); Cell incubator (Thermo, USA), etc.

Study objects and specimens

15 cases of cartilage samples were collected from OA patients receiving total joint arthroplasty in 89th Hospital from January 2014 to January 2015. The study was approved by ethics committee, and all the enrolled patients had signed the informed consent.

Chondrocyte extraction and cultivation

The cartilage specimens collected from patients was washed by PBS for three times and moved to a 90 mm petri dish in biological safety cabinet. The tissue was cut into pieces by ophthalmic scissors and incubated in 7 ml DMEM medium (containing 2% type II collagenase). After cultured for 24 h at 37°C and 5% CO_2, the cells were collected by filtration on 200 mesh strainer and centrifuged at 1000 r/min for 5 min. The left OA chondrocytes were resuspended in 3 ml DMEM complete medium (containing 10% FBS) and transferred to 90 mm petri dish. The cells were maintained in incubator after adding 7 ml DMEM medium.

CCK8 assay

OA chondrocytes in logarithmic phase were digested and collected 1000 r/min 5 min centrifugation. 1×10^4 cells were seeded in 96-well plate at 100 μl volume and divided into five groups, including blank control, DMSO group, and experimental group (low, middle, and high dose groups) with six repeats. IL-6 concentration in experimental group was 20 nm, 40 nm, and 80 nm, respectively. The cells were cultivated for 24 h after seeding, and treated for 12, 24, 36, 48, 60, and 72 h. Then the cells were washed by PBS for three times and added with 100 μl 10% CCK8 for 4 h. At last, the plate was read on microplate reader to obtain the absorbance value and draw the cell growth curve.

Cell transfection

OA chondrocytes in logarithmic phase were seeded in 12-well plate and cultivated for 24 h. The cells were divided into four groups, including NC group, miR-200 mimic group, NC inhibitor group, and miR-200 inhibitor group. 100 μl serum free MEM medium was mixed with 2 μl miRNA mimic as tube A. Another 100 μl serum free MEM medium was mixed with 3 μl lipo2000 as tube B. Tube A and B were mixed and standing at room temperature for 5 min. 800 μl mixture was added to each well for cultivation.

Real-time PCR

Total RNA was extracted from tissue and cells using Trizol. 1 μg RNA was incubated at 65°C for 5 min and reverse transcripted to cDNA according to the manual. 1 μl cDNA was mixed with 1 μl primers, 10 μl qPCR Mix, and water to form 20 μl qPCR reaction solution. Real time PCR was performed on PCR amplifier. The condition was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 72°C for 15 s, and 60°C for 45 s. The primers used were listed in **Table 1**.

Statistical analysis

SPSS 19.0 was applied for statistical analysis. All experiments were repeated for at least three times. The results were presented as mean ± standard deviation. T test or ANOVA were applied for comparison. $P < 0.05$ was considered as statistical significance.

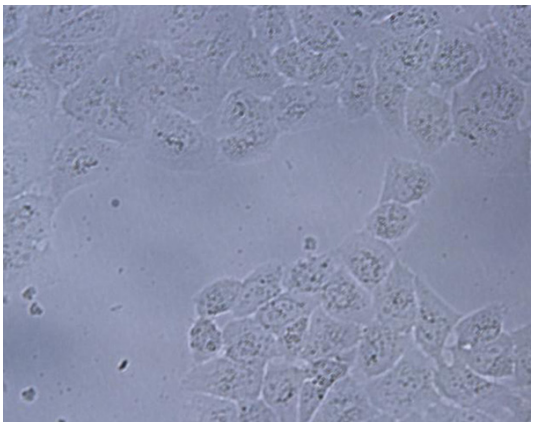

Figure 1. Primary chondrocytes grew adhering to the wall (400×).

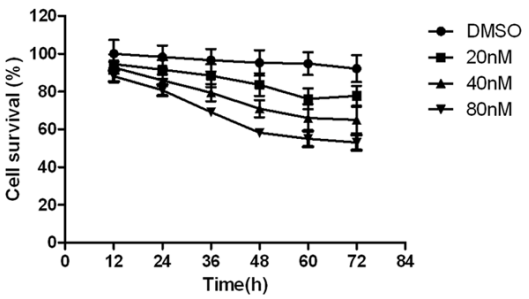

Figure 2. IL-6 impact on chondrocytes proliferation.

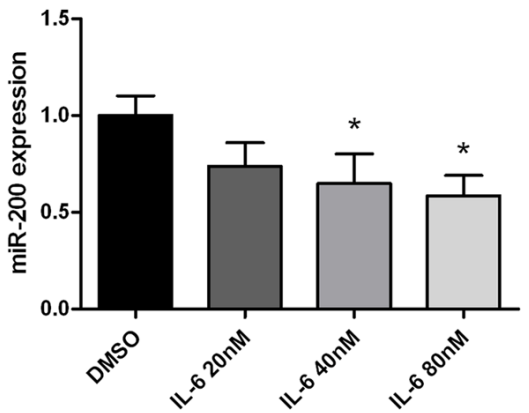

Figure 3. IL-6 impact on miR-200 expression in chondrocytes (*P < 0.05).

Results

Chondrocytes proliferation

We obtained primary chondrocytes by type II collagenase method. Enough primary chondrocytes could be obtained after 24 h cultivation.

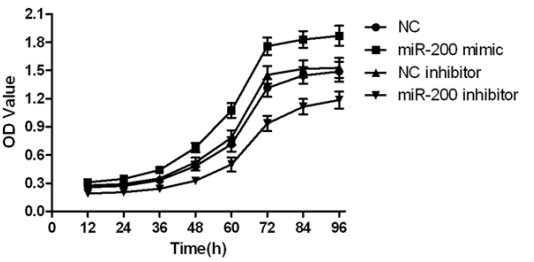

Figure 4. MiR-200 impact on chondrocytes proliferation.

Primary chondrocytes grew adhering to the wall and showed good viability within 72 h (**Figure 1**).

IL-6 impact on chondrocytes proliferation

Primary chondrocytes were divided into five groups, including blank control, DMSO group, and experimental group (low, middle, and high dose groups) with six repeats. IL-6 concentration in experimental group was 20 nm, 40 nm, and 80 nm, respectively. CCK8 was applied to test chondrocytes proliferation. As shown in **Figure 2**, chondrocytes proliferative ability weakened under IL-6 treatment following time elongation. At the same time point, chondrocytes proliferative ability reduced following IL-6 concentration elevation. IL-6 group showed significant difference compared with control ($F=21.47$, $P < 0.05$).

IL-6 impact on miR-200 expression in chondrocytes

Primary chondrocytes were divided into five groups, including blank control, DMSO group, and experimental group (low, middle, and high dose groups) with six repeats. IL-6 concentration in experimental group was 20 nm, 40 nm, and 80 nm, respectively. Real time PCR results revealed that compared with DMSO group, miR-200 expression declined in IL-6 treatment group by 27%, 35%, and 41%, respectively. In certain concentration range, miR-200 decreased following IL-6 concentration enhancement (**Figure 3**).

miR-200 impact on chondrocytes proliferation

The chondrocytes were divided into four groups, including NC group, miR-200 mimic group, NC inhibitor group, and miR-200 inhibitor group. The cells were cultivated for 24 h after seeding,

and treated for 12, 24, 36, 48, 60, and 72 h. CCK8 results showed that compared with NC group, miR-200 mimic group presented cell proliferation increase ($P < 0.05$). Compared with NC inhibitor group, miR-200 inhibitor transfection weakened cell proliferative ability ($P < 0.05$) (**Figure 4**).

Discussion

OA is a type of degenerative disease occurred in the joints caused by various factors in vivo and in vitro. Its clinical manifestation includes joint pain, joint swelling, stiffness, activity limitation, and joint deformity, etc. [12]. OA most occurs in the elderly with the incidence as 5.6% [13]. Its incidence elevated in recent years following aging of population. It also seriously influences patient's physical and mental health, quality of life, and the social medical treatment cost [14]. OA occurrence is a complex biological process, and its pathogenesis is still unclear. Research showed that articular chondrocytes apoptosis and cell matrix degradation play important roles. Recent study found that IL-6 overexpressed in OA patients and had correlation with OA [15]. Animal model showed that IL-6 overexpression in cell matrix was associated with OA cells cartilage proteoglycan synthesis in early stage [16]. Numerous studies showed IL-6 played a critical role in OA, whereas its mechanism still needs investigation.

MiRNAs are kind of highly conservative noncoding small RNA molecules, with the length at about 22 nt. Though miRNAs cannot code protein, it can influence protein expression through a special way. Mature miRNA inhibits gene translation and regulates gene expression by incomplete complementary binding with target mRNA's 3' UTR [17]. MiRNA has multidimensional regulation on target genes. That is a kind of miRNA can regulate multiple target genes, and a target gene can be regulated by various miRNAs [18]. Such network regulation let miRNA can mediate about 30% gene expressions in the body [19]. Previous studies showed that miR-200 low expressed in OA patients [20]. To further investigate the role of miR-200 in OA, we discussed miR-200 impact and possible molecular mechanism in OA chondrocytes proliferation.

This study collected 15 cases of articular cartilage tissues from OA patients receiving joint replacement and obtained primary chondrocytes using type II collagenase method. Primary chondrocytes grew adhering to the wall and showed good viability within 72 h. Previous results showed that IL-6 highly expressed in OA patients, but the mechanism of high concentration IL-6 impact on chondrocytes proliferation is still unclear. CCK8 assay revealed that compared with DMSO control, chondrocytes proliferative ability reduced following the increase of IL-6 concentration, its proliferative ability also decreased following IL-6 treatment time elongation. It suggested that IL-6 can suppress chondrocytes proliferation. Previous study reported that miR-200 expression declined in OA patients, and OA was often companied by articular cartilage damage and cartilage cell proliferation reduction. Is there a relationship between IL-6 overexpression and miR-200 reduction in OA patients? We applied real time PCR to test miR-200 expression in chondrocytes and found that compared with DMSO control, IL-6 exposure can decrease miR-200 level by 35%. MiR-200 expression gradually declined following IL-6 concentration elevation, suggesting that IL-6 can inhibit miR-200 expression in chondrocytes. To further explore the related mechanism, we changed miR-200 expression level to observe chondrocytes proliferative ability variation. CCK8 assay demonstrated that compared with negative control, miR-200 mimic transfection enhanced chondrocyte proliferation, while miR-200 inhibitor transfection weakened chondrocyte proliferative ability. It revealed that miR-200 can affect chondrocytes proliferation.

In conclusion, IL-6 can inhibit miR-200 expression in OA chondrocytes. IL-6 suppresses chondrocyte proliferation by downregulating miR-200 level.

Disclosure of conflict of interest

None.

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