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Effects of osteopontin on the expression of IL-6 and IL-8 inflammatory factors in human knee osteoarthritis chondrocytes

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Abstract. – OBJECTIVE: Osteoarthritis (OA) is a chronic musculoskeletal disease characterized by progressive destruction of articular cartilage, OA lead to chronic pain and functional restrictions in affected joints. The present study was to investigate the role of osteopontin (OPN) in the athogenesis of OA through studying the effect of OPN on expression of IL-6 and IL-8 inflammatory factors in human OA chondrocytes.

PATIENTS AND METHODS: One-step type II collagenase digestive method was used to isolate OA chondrocytes from sectional cartilage specimens of 16 primary knee OA patients received total knee replacement surgery. Synchronized first-generation chondrocytes were then treated with OPN (100 ng/ml or 1 μg/mL). The changes in cell morphology of OA chondrocytes were analyzed before and after treated with OPN; and the expression levels of IL-6 and IL-8 were evaluated by real-time q-PCR.

RESULTS: Chondrocytes were successfully isolated from human OA knee cartilage, and the viability of isolated chondrocytes was 92.11 ± 3.13%. Adherent chondrocytes formed clusters of irregular polygonal shape with intercellular pseudopodia extension. After OPN treatment, cells became fusiform or irregularly shaped, and the number of intercellular pseudopodia decreased significantly. The mRNA expression of IL-6 increased to 1.83 times at 0.1 μg/ml of OPN and 3.1 times at the dose of 1 μg/ml; the expression of IL-8 increased to 1.57 and 3.27 times at the dose of 0.1 μg/ml and 1 μg/ml respectively.

CONCLUSIONS: OPN could up-regulate expression of IL-6 and IL-8 cytokines in human OA chondrocytes, and the expression increased with the increasing concentration of OPN, which might be one of the potential mechanisms of OPN in the development of OA.

Key Words:
Osteoarthritis (OA), Osteopontin (OPN), Chondrocytes, Interleukin-6 (IL-6), Interleukin-8 (IL-8).

Introduction

Osteoarthritis (OA) is a disease characterized by pathological degenerative changes in articular cartilage, sclerosis of subchondral bone and synovitis[1]. It is estimated that about 10% of men and 18% of women have symptomatic OA, the number reach to 60-65% in old aged over 60 years, OA lead to limitations of movement world widely[2]. Although clinical researches of OA have been investigated extensively, the etiology of this disease remains elucidated poorly. OA is a condition of "diverse etiology" with various predisposing factors, and several biochemical and biomechanical factors are involved in the pathogenesis.

Currently, inflammation is believed to involve in the development and progression of OA at the early stages[3]. Emerging experimental evidence demonstrated that secreted inflammatory factors, such as proinflammatory cytokines were critical mediators of the disturbed metabolism and enhanced catabolism of OA. Osteopontin (OPN), functions as a proinflammatory cytokine, plays a critical role in the regulation of tissue repair and remodeling[4]. Expression of OPN during chondrocyte maturation is one of the important events involved in cartilage-to-bone transitions in fracture repair[5,6]. Researches have demonstrated that existing of OPN in plasma, synovial fluid and articular cartilage was associate with progressive joint damage, and OPN could serve as a biochemical marker to determine the severity and progression of knee OA[2,7].

As a pleiotropic cytokine with various biologic activities, interleukin-6 (IL-6) plays important role in the pathogenesis of OA[8]. Researches indicated that expression of IL-6 levels in synovial fluid was directly correlated with the stage of OA[9,10], and higher baseline value of IL-6 predict greater risk of cartilage loss in OA[11]. IL-8 is a

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member of the alpha chemokine family of cytokines, which originally identified as a neutrophil chemoattractant[12]. IL-6 was correlated with Interleukin 8 (IL-8) in synovial fluid of various arthropathies, both IL-6 and IL-8 were associated with inflammation, they all involved in the cartilage degradation process of OA[8,13].

Although expressions of OPN and cytokines IL-6, IL-8 have been investigated in patients with knee OA, few researches were performed to study the influence of OPN on the expressions of IL-6 and IL-8 in OA. Present study mainly focused on the effect of OPN on the expression of IL-6 and IL-8 after OPN-treated OA chondrocytes *in vitro*. We hope could provide theoretical basis for new targets of OA treatment.

Patients and Methods

Patients

The present study was approved by Ethical Committee of XiangYa Hospital, Central South University of China, and was conducted in accordance with the Declaration of Helsinki. Full ethical consent was obtained from all patients. Biopsies of articular cartilage were obtained from 16 OA patients (12 women, 4 men) with primary OA who received knee replacement surgery at our hospital in 2012. They were 42-75 years old with an average age of 67 ± 4.83.

OA was diagnosed according to the guide for Diagnosis and Treatment of Osteoarthritis established by Branch of Orthopedics and Traumatology, Chinese Medical Association, as described by Fu and Zhang[14]. The detailed diagnostic criteria including: (1) repeated knee pain for a period of 1 month; (2) joint space narrowing, sclerosis or cystic change in subchondral bone; (3) clear and sticky synovial fluid, WBC < 2.000/ml; (4) age at least 40; (5) morning stiffness at least 3 min, and friction fremitus following physical activity. Clinical data were carefully reviewed; patients who have secondary OA and inflammatory joint diseases such as rheumatoid arthritis (RA), suppurative arthritis were excluded.

Isolation and Culture of the OA Chondrocytes

Articular cartilage specimens were minced and digested with 0.15% type II collagenase (Abcam, Cambridge, MA, USA) for 6 h at 37°C with 5% CO_2. Digested cartilage tissue was transferred into clean 15-mL tubes and centrifuged at 1,000 rpm for 8 min. Pellet was resuspended in DMEM/F-12 supplemented (GIBCO, Waltham, MA, USA) with 20 % fetal bovine serum (FCS, GIBCO, USA) to obtain OA chondrocyte suspension.

Immunocytochemistry and OPN intervention

An immunocytochemical Kit (Mai-xin Bio, Fuzhou, China) was used to stain collagen II antigen of cartilage cells in a tissue sample according to the manufacturer's manual.

To synchronize the cells, first-generation OA chondrocytes were cultured in DMEM supplemented with 5% FCS for 24 h. Synchronized chondrocytes were treated with 0.1 g/mL (group B) or 1 g/mL (group C) OPN in DMEM supplemented with 20% FCS, or left untreated (control group, group A), and then cultured at 37°C with 5% CO_2 for 48 h. Cells were observed using inverted microscope (Olympus Corporation, Japan).

RNA Isolation

After treated by OPN for 48 h, total RNA was extracted from chondrocytes by TRIZOL (Life Technologies, Carlsbad, CA, USA), according to manufacturer's instruction. Briefly, 1 mL of TRIZOL was added to the cells, and cell lysates were transferred into 1.5 mL micro centrifuge tube and incubated at room temperature for 5 min. 0.2 mL of chloroform was added then. The solution was vigorously mixed for 15 s, incubated at room temperature for 3 min and centrifuged at 10,000 g for 10 min. The supernatant was incubated with 0.5 mL of isopropyl alcohol for 10 min at room temperature, followed by centrifugation at 10,000 g for 10 min. Supernatant was carefully removed, and the pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500 g for 10 min. Pellet containing RNA was dried for 5 min, resuspended in DEPC and kept at 4°C over night.

First-Strand cDNA Synthesis

First-strand cDNA was synthesized (Table I) from isolated RNA with Oligo (deoxythymidine) [Oligo(dT)] 18 primer using RevertAid™ M-MuLV Reverse Transcriptase (Thermo scientific, Waltham, MA, USA) and with Ribo-Lock™ Rnase Inhibitor (Thermo scientific, Waltham, MA, USA). RNA was retro transcribed for 1 h at 42°C, and the reverse transcriptase activity was y inactivated at 70°C subsequently.

Table I. Material used in the RT-PCR.

Reagent	Volume
59 RT reaction buffer	4 μL
RiboLock™ Rnase Inhibitor (20 μ/μL)	1 μL
10 nM dNTP Mix	2 μL
RevertAid™ M-MuLV reverse transcriptase (200 μ/μL)	1 μL
Water, nuclease-free	Up to 20 μL

Real-Time Q-PCR Determining the mRNA Expressions of IL-6 and IL-8

The primer pair for IL-6 was made to bp 42-61 (sense) and bp 334-354 (antisense) according to IL-6 cDNA sequence, and the IL-8 primer pair was made to the bp 147-174 (sense) and bp 342-366 (antisense). Real-time PCR was performed in a final volume of 25 L containing 500 ng cDNA, 0.3 M of each primer, and 12.5 L Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, Waltham, MA, USA) using 7,500 real-time PCR system (Applied Biosystems Life Technologies, Carlsbad, CA, USA). The samples underwent initial denaturation at 95°C for 10 min, followed by 40 cycles consisting of the following steps: 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Statistical Analysis

All data were analyzed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and presented as mean ± SD. *T* test was used for comparison between groups. The test level was set as a = 0.05. The difference was considered statistically significant at $p < 0.05$.

Results

Cell Number and Viability of the Isolated OA Chondrocytes

Cell counts were determined by hemocytometer, the viability of isolated chondrocytes was 92.11 ± 3.13% directly after isolation (Figure 1) when measured by the trypan blue dye exclusion test in accordance with the requirements for further processing.

Morphological Changes of OPN-Treated OA Chondrocytes

Following primary adhesion, chondrocytes migrated and formed clusters of irregular polygonal shape (Figure 2A). Some chondrocytes clusters exhibited lower cell density with fibroblast-like morphology with intercellular pseudopodial extensions (Figure 2B and C). After treated by OPN, most chondrocytes exited in fusiform or of irregular shape, while the amount of intercellular pseudopodia significantly decreased (Figure 2D).

Immunohistochemistry Analysis of OA ChonDrocytes

So far, there is no unique and effective method to distinguish the normal chondrocytes and OA chondrocytes. Based on the current knowledge, the chondrocytes from the edge of articular surface of the exposed bone is considered to be OA chondrocytes, and OA chondrocytes generally has high expression of collagen II[15]. Immunocytochemistry showed that OA chondrocytes cultures were stained positively for collagen II. A large number of OA chondrocytes were identified in cytoplasm and matrix of cartilage cell (Figure 3).

Effects of OPN on the Expression of IL-6 and IL-8 in OA Chondrocytes

After treated by 48 hour, the expression of IL-6 and IL-8 mRNA in OA chondrocytes (group B and group C) in vitro increased significantly as compared with control (group A) ($p < 0.05$). The increased IL-6 and IL-8 levels were correlated with the increased concentration of OPN (Tables

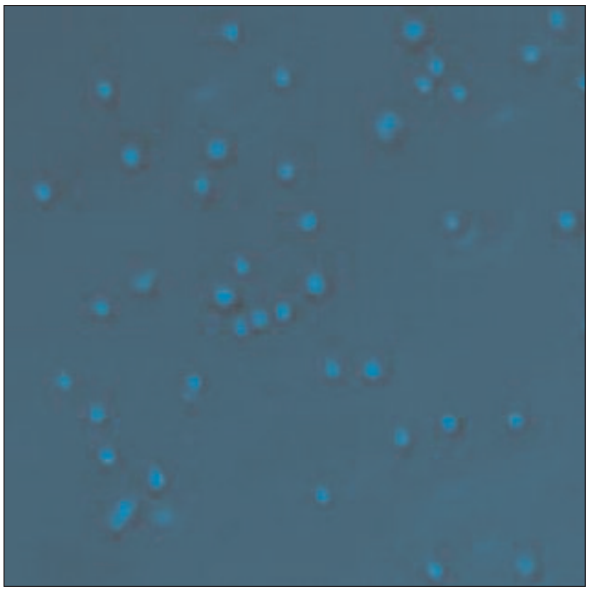

Figure 1. Trypan blue dye staining of chondrocyte cells immediately after isolation (×100).

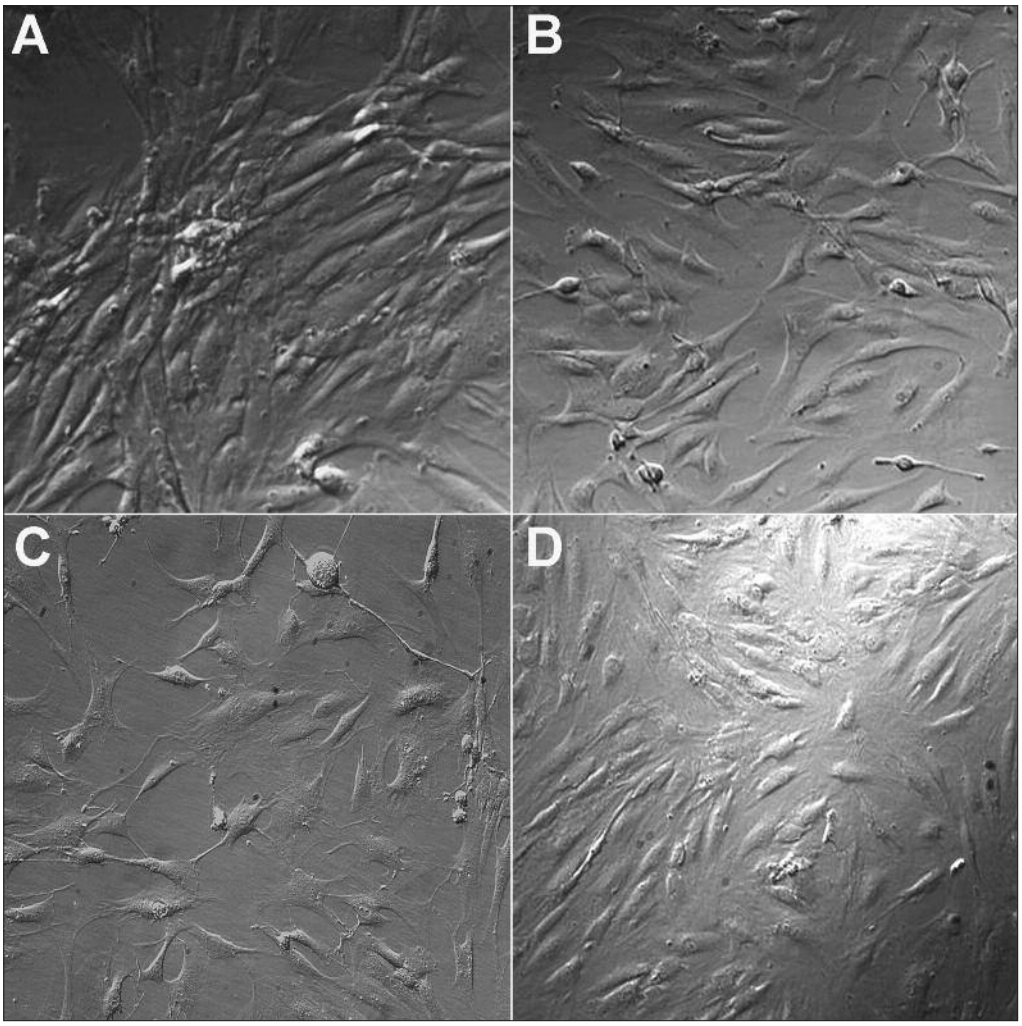

Figure 2. Cellular morphology of the chondrocyte cultures at the different incubation time. ***A,*** Primary OA chondrocytes (×200). ***B,*** First-generation OA chondrocytes (×200). ***C,*** First-generation OA chondrocytes (×400). ***D,*** OA chondrocytes after OPN intervention (×200).

II). The expression of IL-6 and IL-8 was positively related to OPN concentration ($r = 0.59$, $p < 0.01$, and $r = 0.52$, $p < 0.01$, respectively).

Discussions

OA is a common chronic arthritis disease, which caused joint pain and deformity. Currently, the mechanism of OA has not been interpreted clearly, but more and more evidence points to a critical role of cytokines in the pathophysiology of OA[16]. The integrity of articular cartilage is maintained by the balance between cytokine-driven anabolic and catabolic processes. Unregulated of these molecules are believed to participate in the pathophysiology of many joint diseases. High levels of cytokines, including interleukin (IL)-1, IL-6 and IL-8 have been found in various arthropathies, which suggest that they may play important role in the pathogenesis of these disease[12]. Studies have demonstrated that IL-6 is involved in inflammatory of degenerative joint diseases in the presence of a soluble form of IL6 receptor (sIL6R). Chondrocytes produce low levels of IL6 normally, but expression of IL6 and sIL6R increased in synovial fluid and sera in OA patients [10]. Deficiency of IL6 was associated with a decreased number of inflammatory cells in knee joints of mice, and further lead to reduced response to collagen-induced arthritis[17]. IL-6 levels in the synovial fluid seemed to directly correlate with the stage of OA[9,10], and the higher baseline values of IL-6

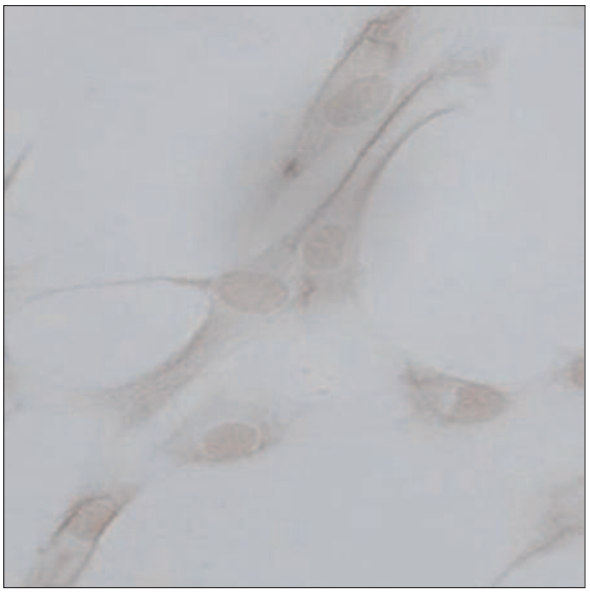

Figure 3. Immunocytochemical staining of collagen II in OA chondrocyte cultures (×400).

could predict greater risk of cartilage loss in OA[11]. IL-6 play an important role during the pathogenesis of OA[8,13].

IL-8 is also known as an osteoclastogenic factor which involved in osteoclastogenesis and bone resorption process, IL-8 also contributed to the increased osteolysis in metastatic bone disease[18]. There was a correlation between IL-6 and IL-8 levels in synovial fluid of various arthropathies[12]. Both IL-6 and IL-8 were associated with inflammation, and they were involved in the cartilage degradation process of OA[8,13].

Osteopontin is a soluble phosphoprotein secreted by activated T lymphocytes, macrophages, osteoclasts, and hypertrophic cartilage cells. OPN play important role in number of physiological and pathological processes such as entochondrostosis, inflammatory reaction of joint, and osteoporosis. OPN involved in the molecular pathogenesis of OA and contributes to progressive degeneration of articular cartilage[19]. OPN-null mice were protected against inflammatory joint destruction in collagen-induced arthritis[20]. A blocking antibody directed against the thrombin-cleaved neoepitope of OPN also showed a curative effect on induced arthritis in monkeys[21]. Deposition of OPN protein and mRNA expression increased the severity of matrix degradation in human osteoarthritic cartilage[10]; in addition, advanced OA cartilage demonstrated significantly higher OPN mRNA expression than the minimal OA cartilage[22]. For patients with OA, OPN was detected strongly in the synovium and synovial fluid of inflamed joints, these cumulative researches suggested that OPN was associated with progressive joint damage[2,7].

An *in vitro* study[23] on the function of OPN in arthritis have revealed that OPN stimulated the production of several proinflammatory cytokines in patients with RA. Take et al[24] found that specifically modified OPN in RA fibroblast-like synoviocytes enhanced production of IL-6. Furthermore, OPN has also been reported to correlate with the expressions of IL-6 and IL-8 in other diseases. Tanaka et al[25] indicated that OPN treatment substantially up-regulated the production of IL-8 by HUVEC. OPN may activate a novel monocyte-mediated mechanism of neovascularization triggered by IL-1β, which could significant increases the secreted levels of IL-1β, as well as the proinflammatory/proangiogenic mediators TNF-α, IL-8, and IL-6 in human monocytes[26]. IL-lβ has been confirmed to play a central role in severe arthropathies, and the response of chondrocytes obtained from OA patients to IL-1β were distinct[12,27]. In OA patients, IL-1β, IL-6, TNF-α and IL-8 coexisted in the synovial fluid. IL-8 has been reported to enhance the releases of IL-1β, IL-6 and TNF-α in low concentration, while a higher dose of IL-8 conversely suppressed release of these cytokines from damaged cells[28]. All these factors may work together to exert influences of OPN on the cartilage degradation of OA.

Table II. Relative levels of IL-6 mRNA and IL-8 mRNA after OPN treatment

Group	Relative mRNA level (mean ± SD)	
	IL-6	IL-8
A (Control group)	1	1
B (0.1 μg/mL OPN treatment)	1.84 ± 0.63*,#	1.58 ± 0.54*,#
C (1 μg/mL OPN treatment)	3.12 ± 1.23*	3.29 ± 1.77*

*$p < 0.05$ vs the group A; #$p < 0.05$ vs the group C.

Conclusions

We investigated the gene expression pattern of IL-6 and IL-8 in OA chondrocyte culture after OPN intervention. Our research showed that after treated by OPN 48 h, both IL-6 and IL-8 expression in OA chondrocytes increased significantly ($p < 0.05$). This increasing was directly dependent on OPN concentration, which suggest that OPN could up-regulate the expression of IL-6 and IL-8 in OA chrondrocytes in a dose-dependent manner, it may shed light on the potential mechanisms of OPN on the development of OA.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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