Original Article
Osteopontin can decrease the expression of Col-II and COMP in cartilage cells in vitro

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Abstract: Objective: To observe the effect of osteopontin (OPN) on the expression of collagen type II (Col-II) and oligomeric matrix protein COMP in cartilage cells of knee osteoarthritis, and explore the mechanism of OPN in the knee osteoarthritis. Methods: Cartilage cells were isolated from fetal rabbit, they were divided into 3 groups: A (control); B (0.5 µM OPN) and C (1 µM OPN). The expression levels of Col-2 and COMP were detected by RT-PCR and Western blotting methods. MMP-13 was detected using ELISA. The proliferation was determined by MTT method. Results: Compared with the group, the expression levels of Col-2 and COMP in cartilage cells decreased in the intervention group with dose dependent ($P<0.05$). The expression of MMP-13 in culture supernatant and the proliferation increased in the intervention group ($P<0.05$). Conclusion: OPN can down-regulate the expression levels of Col-2 and COMP in cartilage cells and up-regulate the expression of MMP-13 in culture supernatant and promote the proliferation of cells, which could accelerate the pathological process of cartilage cells.

Keywords: Osteopontin, osteoarthritis, Col-2, COMP

Introduction

The occurrence and development of knee osteoarthritis was closely related with the microenvironment of osteoarthritis, the components of extracellular matrix regulated the microenvironment directly. Therefore the extracellular matrix proteins were crucial to the occurrence and development of osteoarthritis. Recently, some extracellular matrix proteins such as periostin [1], MMP [2] and osteopontin (OPN) [3] were found to play important roles in promoting the inflammatory occurrence of cartilage cells in knee osteoarthritis, especially the OPN protein has attracted wide attention. As an important extracellular matrix protein, OPN can mediate cellular growth, survival, adhesion and migration in osteoarthritis [4, 5]. Although we have found that OPN highly expressed in many inflammatory diseases, even some researchers thought that OPN can be used as a biomarker for the diagnosis of knee osteoarthritis [6, 7], but the mechanism of OPN in the knee osteoarthritis remains unclear. Studies have reported that OPN promoting the occurrence and development of knee osteoarthritis may be associated with the activation of some proteases such as MMPs [7] and some growth factor

receptors such as EGFR [3] and so on. These studies sufficiently indicated that OPN most likely played a key role in many arthritis diseases including knee osteoarthritis.

In this study, we observed the effect of OPN on the expression of collagen type II (Col-II) and oligomeric matrix protein COMP in cartilage cells of knee osteoarthritis, and explored the mechanism of OPN in the knee osteoarthritis.

Materials and methods

Isolation and microcarrier culture of cartilage cells in vitro

Briefly, articular cartilage tissues were taken from New Zealand rabbits. The tissues were cut into small pieces and cultured with DMEM/F12 medium containing 20% FCS at 37°C with 5% CO_2 and 95% relative humidity.

Biosilon microcarrier beads were washed with PBS and autoclaved. The beads were suspended in DMEM medium containing 10% FCS, 1×10^5 cells/mL cartilage cells were seeded in the 100 mL rotating bottle with 10 g/L concentrations of Biosilon micro carrier, volume of culture medium was 50 mL. The bottle was placed
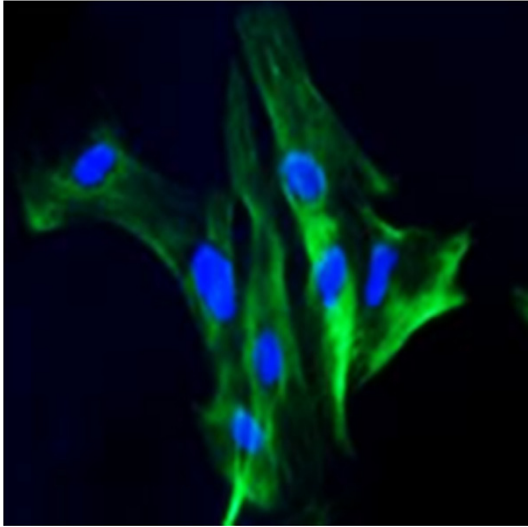
Figure 1. Immunofluorescence staining results of cartilage cells (×400).

Table 1. Survival rate of cartilage cells ($\overline{X}$ ±SEM)

Group	24 h	48 h	72 h
Control	94±1.0	94±0.8	93±1.0
0.5 µM OPN	94±1.2	93.7±0.6	92.1±1.7
1 µM OPN	93±0.6	94.4±1.5	92.4±1.0

in the incubator at 37°C with 5% CO_2, 95% relative humidity and speed of 30 rpm.

Immunofluorescence identification of cartilage cells

The fourth generation cells in good growth status were washed with PBS for 3 times and fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature. They were washed with PBS for 3 times and incubated with 10% goat serum for 1 h at room temperature to block nonspecific binding. Then they were washed with PBS for 3 times and first antibodies were added (1:200) and incubated at 4°C overnight. After that they were washed with PBS for 3 times and FITC antibodies were added (1:128) and incubated at dark for 1 h, the nucleus was stained with DAPI. Mounted the slides and observed under the fluorescence microscope with photographic record.

Expression of Col-2 and COMP were detected with RT-PCR

Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen) according to the man-

ufacturer's protocol. 1 µg total RNA was subjected to reverse transcription using reverse transcription system and Real-time PCR were performed using SYNBR Green PCR Master Mix following the manufacturer's instructions. All values obtained were normalized to GAPDH. The PCR primers were as follows: Col-2F: 5'-TGCCCAGAAAATGAAAAAGG-3', R: 5'-GTGTAT-GTGGCAATGCGTTC-3'; COMP F: 5'-GAGAACTTT-GCCGTTGAAGC-3', R: 5'-GCTTCCTGTAGGTGG-CAATC-3'. Following amplification for 35 cycles, the products were detected using 1.5% agarose gel electrophoresis and viewed with digital gel imaging system.

Western-blotting

Total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. The membrane containing the proteins was used for immunoblotting with required antibodies. They were blocked with 5% non-fat milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20) for 2 h, then incubated with the primary antibodies: rabbit anti-Col-2antibodies (1:200), rabbit anti-COMP antibodies (1:200) and anti-GAPDH (1:5000) at 4°C overnight. Then they were incubated with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Antibody binding was detected using ChemiDoc XRS Imaging system.

MMP13 detection with ELISA

The expression of MMP13 was detected according to the manual of ELISA kits. Briefly, A total of 50 µl cell supernatant and 50 µl sample dilution were added into 96 well plate coated with specific antibody and incubation at 37°C for 2 h. The plates were washed 5 times, 100 µl substrate of enzyme reaction was added and incubation at 37°C for 2 h. Then plates were washed 5 times and 100 µl chromogenic reaction liquid was added and incubation at 37°C for 30 min, the termination liquid was added to terminate the reaction. The absorbance was detected using microplate reader at 450 nm wavelength.

Proliferation was detected with MTT and BrdU method

MTT: Briefly, the primary cartilage cells in good growth condition were seeded in 96-well plates

Table 2. Effects of OPN on the expression levels of Col-2 and COMP ($\overline{X}$±SEM)

	Group	24 h	48 h	72 h
Col-2	Control	8.12±2.10	8.51±2.31	10.14±2.18
	0.5 µM OPN	10.75±2.89	14.11±3.751*	25.63±8.791*
	1 µM OPN	15.33±4.851*	22.16±7.141*	45.86±9.251*
COMP	Control	6.35±0.80	7.18±2.89	7.32±1.02
	0.5 µM OPN	12.04±2.89	16.44±1.051*	26.23±0.621*
	1 µM OPN	20.56±1.071*	34.44±2.101*	50.02±2.301*

*Compare with control group, $P < 0.05$.

at a density of 1×104 cells/well followed by incubation at 37°C with 5% CO_2 in a humidified atmosphere. The medium was discarded and different concentrations of OPN diluted with medium were added into the wells after incubation for 24 h. Then 10 µl 0.5% MTT were added to these wells. After incubation for 4 h, the supernatant was discarded and 100 µl DMSO was added to these wells. The OD values were measured at 590 nm wavelength.

Brdu: Briefly, the primary cartilage cells in good growth condition were seeded in 96-well plates at a density of 1×104 cells/well and making majority of cells in G0 phase with 0.4% FCS. They were terminated with Brdu (3 µg/L) after culture for 3 days and incubated at 37°C with 5% CO_2 for 40 min. They were washed with PBS for 3 times and fixed with methanol/acetic acid for 10 min. Endogenous oxidase was inactivated with 0.3% H_2O_2-methanol for 30 min and blocked with 5% rabbit serum. Nucleic acid was denatured with formamide at 100°C for 5 min. Brdu antibody was added and the OD values were measured at 490 nm wavelength.

Statistical analysis

Data are presented as $\overline{X}$±SEM. The SPSS software package 19.0 was used for the statistical tests. t-test was used to compare between two groups. $P<0.05$ was considered statistically significant.

Results

Immunofluorescence identification of cartilage cells

The Immunofluorescence staining result of cells was shown in **Figure 1**. All of cartilage cells were positive and stained as green fluorescence.

Survival rate detection

Cartilage cells were counted with 0.4% trypan blue staining methods after culture for 24 h, 48 h and 72 h treated with OPN (**Table 1**). There was no obvious effect on the cellular growth ($P>0.05$).

RT-PCR results of the expression of Col-2 and COMP

The expression levels of Col-2 and COMP after treated with OPN for 24 h, 48 h and 72 h were detected and the results were shown in **Table 2** and **Figure 2**.

Compared with control group, the expression levels of Col-2 and COMP decreased significantly in 0.5 µM OPN group after 48 h ($P<0.05$) and in 1 µM OPN group after 24 h ($P<0.05$).

Western-blotting results of Col-2 and COMP

The Col-2 and COMP proteins after treated with OPN for 24 h, 48 h and 72 h were detected and the results were shown in **Figure 3**.

Compared with control group, The protein levels of Col-2 and COMP decreased significantly in 0.5 µM OPN group after 48 h ($P<0.05$) and in 1 µM OPN group after 24 h ($P<0.05$).

Detection of MMP-13

As shown in **Table 3**, the expression levels of MMP-13 increased after treated with OPN for 48 h, ($P<0.05$), and the expression level of MMP-13 increased more along with the increased concentration of OPN intervention.

Effect of OPN on the proliferation of cartilage cells

The MTT results were shown in **Figure 4A**. There were significant differences between control group and OPN treated group ($P<0.05$). 1 µM group can promote cell growth more than 0.5 µM group. The Brdu results were shown in **Figure 4B**, there was no significant difference between control group and OPN treated group after culture for 24 h. There was significant difference between control group and OPN treated group after culture for 48 h ($P<0.05$).
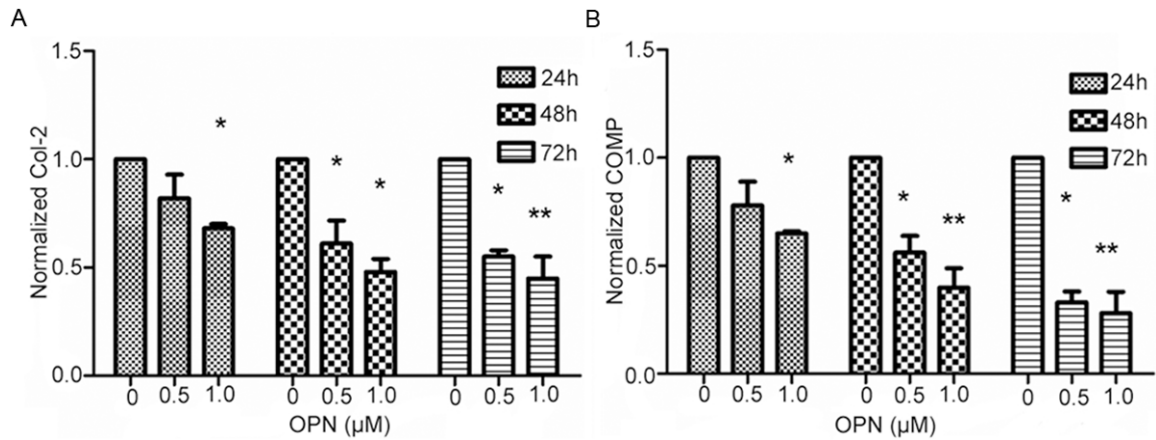
Figure 2. RT-PCR results of the expression of Col-2 and COMP. A. Normalized Col-2; B. Normalized COMP.
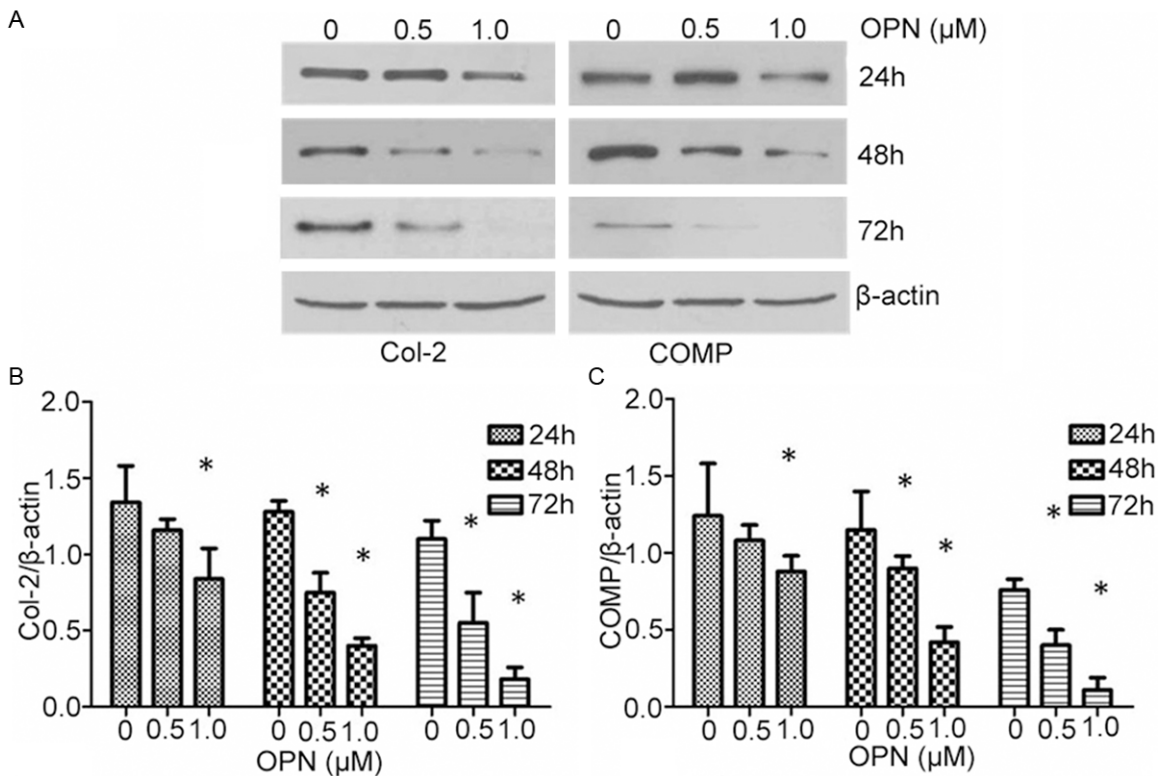
Figure 3. Western-blotting results of Col-2 and COMP. A. Western-blotting results; B. Normalized Col-2; C. Normalized COMP.

**Table 3.** Effects of OPN on the expression levels of MMP-13 ($\overline{X}$±SEM)

Group	MMP-13 (µg/ml)		
	24 h	48 h	72 h
Control	70.55±12.73	66.88±13.02	68.48±12.50
0.5 µM OPN	75.66±14.03	79.45±15.10*	83.66±9.84*
1 µM OPN	80.45±12.56	87.98±14.65*	89.44±8.96*

*Compare with control group, $P < 0.05$.

Discussion

Knee osteoarthritis is a common joint disease of orthopedics, most of the patients are elderly. One of the main reasons which cause joint dysfunction and pain is the defect of articular cartilage. Articular cartilage has limited self repair capacity after injury due to the lack of effective differen-
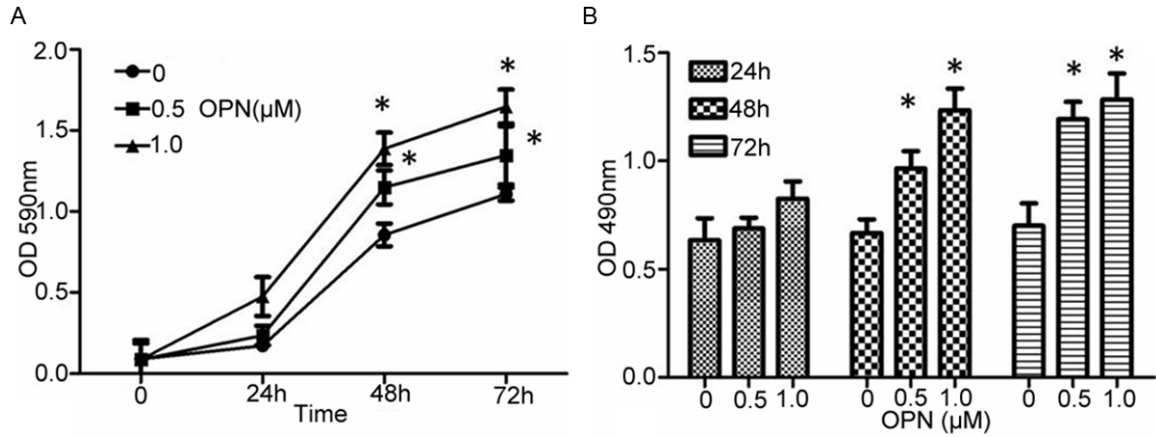
Figure 4. Effect of OPN on the proliferation of cartilage cells. A. MTT results; B. Brdu results.

tiation and proliferation ability [7]. Articular cartilage defect patients accounted for about 57.6% of clinical osteoarthritis [8]. The repair of articular cartilage is typical fibrous cartilage repair, which is temporary without adequate mechanical properties [9, 10]. Therefore, how to repair the damage and defect of articular cartilage has become a hot research topic in experimental and clinical medicine in the world.

Articular cartilage is composed of cartilage cells and the extracellular matrix. Cartilage cells easily lead to unstable differentiation after long term culture in vitro, they differentiate into fibroblast like cells and lose the ability to express Col II and secretion of cartilage matrix [9]. We used the cartilage cells of fetal rabbit in this research, which have the developmental plasticity high fecundity and the ability to repair articular cartilage. Micro carrier is a commonly used cell culture carrier, it not only can maintain the phenotype of cartilage cells, but also can increase the culture area. We obtained a large number of high-quality cartilage cells using microcarrier culture technology in this study. The survival rate of cartilage cells obtained by this method was found to be high by viable cell count and activity and phenotype of cells were not affected. This culture method is simple which could provide a reference for the future large-scale culture of cartilage cells in vitro.

OPN played an important role in the pathological processes of osteoarthritis [11, 12]. OPN highly expressed in the joint synovium and synovial fluid of osteoarthritis patients and the expression level was positively related to the degree of arthritis severity [13-15]. OPN may

mediate the activation of inflammatory factors by a variety of signaling pathways such as NF-κB to participate in the pathological process of osteoarthritis [16-18].

Col-II can mediate the cell-cell adhesion and the interaction between the cell and extracellular matrix (such as collagen, fibronectin and laminin) [19, 20]. After Col-II binding to ligand, signal can be transmitted into the cell interior thereby affecting the reorganization of cytoskeleton, phosphorylation of protein or genes expression [21]. Thus Col-II plays important roles in many biological processes such as cell movement, adhesion, synaptogenesis, proliferation, apoptosis and inflammatory reaction [22]. Recent studies suggest that activated Col-II can obviously promote the growth of cartilage cells, and promote regeneration of cartilage cells in the surrounding environment which is not conducive to the growth [23, 24]. Regulation of osteoclasts is a key problem for the treatment research of knee osteoarthritis, while the COMP may have an important role in regulating the differentiation and function of osteoclasts.

In this study we found that the expression of Col-II and COMP and the intervention concentration of OPN were dose-dependent in cartilage cells. The expression and protein levels of Col-2 and COMP decreased with the increase of OPN intervention concentration.

With the exacerbation of knee osteoarthritis, the expression of OPN increased which leading to the decrease of Col-2 and COMP and was not conducive to the repair of cartilage. Further studies found that the intervention of OPN can up-regulate MMP-13 level, while MMP13 can

degrade Col-2. So the intervention of OPN may reduce the expression of Col-II and COMP by up-regulate MMPs, which leading to the destruction of the balance between cartilage matrix synthesis and degradation and osteoarthritis occurred. We also found that OPN could promote the proliferation of cartilage cells in vitro. Dorotka R found that there was a positive correlation between the proliferation of cartilage cells and advances in OA [24]. Therefore, our results further verified that OPN intervention can promote the proliferation of cartilage cells thereby accelerating cartilage degeneration. OPN, Col-II and COMP may become new targets in the treatment of knee osteoarthritis.

Acknowledgements

Disclosure of conflict of interests

None.

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