Co-culture with synovial tissue in patients with rheumatoid arthritis suppress cell proliferation by regulating MAPK pathway in osteoblasts

Weiwei Zheng*, Xueping Gu*, Dan Hu, Yuefeng Hao

Department of Orthopaedics, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou 215008, PR China. *Co-first authors.

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Abstract: There is growing evidence that synovial tissue affects osteoblasts although the mechanisms behind the aberrant bone metabolism in rheumatoid arthritis (RA) are unclear. The aim of this study is to preliminarily establish a co-culture system of rheumatoid arthritis-derived synovial tissue (RAS) and osteoblasts in vitro and to investigate the potential mechanism of RAS on osteoblasts. A consistent volume of approximately 85 mm$^3$ of RAS was cultured isolated and co-cultured with Hfo1.19 cells for up to 21 days. Equal volume of normal synovial tissue (NS) was co-cultured as a control group. Cell proliferation, cell cycle and bone markers were valued and the mechanisms underlying MAPK pathway have been fully delineated. Our findings suggested that co-cultures with RAS exhibited decreased proliferation of Hfo1.19 cells. Moreover, gene and protein expressions of GLUT3 in cells were suppressed, and the cell cycle was also down-regulated. The expressions of related proteins of MAPKs (JNK and p38) signaling pathway were found to be inhibited. Rescue experiments demonstrated that co-cultures with RAS could decrease the growth and cell cycle of Hfo1.19 cells, which were reversed by p-JNK and p-p38 over expression. In conclusion, this study suggested that synovial tissue in patients with RA may negatively regulate osteoblasts proliferation by declining MAPK pathway.

Keywords: Synovial tissue, rheumatoid arthritis, osteoblasts, MAPK pathway

Introduction

Articular bone erosion followed by rheumatoid arthritis (RA) is a hallmark of joint destruction and progressive disability that is linked to poor functional outcomes [1, 2]. Hence there is an urgent need to develop novel targets for bone erosion therapy. Although the precise aetiology of RA remains elusive, abundant evidence has suggested that pathological change of synovium plays a critical role in the pathophysiology of RA [3, 4]. Bone erosion represents localized bone loss resulting from an imbalance in which bone resorption by osteoclasts is more active over bone formation by osteoblasts [5, 6]. Understanding the mechanisms that define the formation of bone erosions requires insight into the interaction of synovium tissue with osteocytes in RA patients.

In vitro models allow exploration of potential interactions between tissue and cells, and have been useful for studying effects of synovial tissue and bone cells on articular bone metabolism. However, most investigations target only synovial fibroblasts or bone cells, which is extremely disparate from RA conditions in vivo [7, 8]. Prior studies have noted that synovium tissue was invaded by inflammation and experienced complex pathological processes when rheumatoid arthritis occurred, including synovial fibroblasts and macrophages [9-11]. Therefore, synovial tissue needs to be considered as an organized whole when studying the effects of synovium on osteocytes. Hence, it is vital to develop a straightforward and reliable platform to assay the interaction of synovium tissue with bone cells to study the underlying mechanisms of bone erosion in RA patients. In the present study, we constructed a co-culture system of synovial tissue and osteoblasts in vitro and the interactions were further investigated.
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Table 1. Patient characteristics

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<th>Age</th>
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<th>Aggravation (months)</th>
<th>Fever</th>
<th>Morning numb</th>
<th>RF (IU/ml)</th>
<th>CRP (mg/L)</th>
<th>ESR (mm/h)</th>
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Notes: RA, rheumatoid arthritis; N, normol; RF, rheumatoid factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ASO, Antistreptolysin-O.

Materials and methods

Hfob1.19 cells culture

Hfob1.19 cells purchased from BioHermes Co., Ltd. (Shanghai, China), and were cultured in Dulbecco’s modified Eagle medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (ProSpec-Tany TechnoGene, Ltd.), and were maintained in a 5% CO₂ incubator at 37°C. The culture medium was replaced according to cell growth. The medium was replaced twice a week and the cells were passaged at confluence of 80%.

Tissue harvest and co-culture

This study was approved by the institutional review board of Suzhou Hospital Affiliated to Nanjing Medical University (NO. IRB20180321). The informed consent were signed by all patients. Synovial tissue of six patients with rheumatoid arthritis and normal synovial tissues from five patients with acute cruciate ligament injury were collected by arthroscopy (Table 1). Patients with rheumatoid arthritis matching the 2010 ACR/EULAR criteria for RA [12], with symptomatic knee synovitis for at least six months (despite adequate medical treatment), treated with arthroscopic synovectomy of the knee were included.

Synovial tissue was excised from each knee joint, and the pathological observation was shown in Figure 1. Tissues were immediately separated and cut into pieces to fill a 6 mm diameter, 3 mm deep well to obtain a consistent volume of approximately 85 mm³. The synovial membrane explants were washed with phosphate buffered saline (PBS, Lonza, Basel, Switzerland) three times and were randomly allocated to one of two experimental groups (n=6 explants/group): 10⁵ Hfob1.19 cells with normal synovial tissue (Hfob1.19+NS), 10⁵ Hfob1.19 cells with rheumatoid arthritis-derived synovial tissue (Hfob1.19+RAS). Tissue and cells were co-cultured in the Dulbecco’s Modified Eagle Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 1% L-glutamic acid and 1% double antibiotics (HyClone, South Logan, UT, USA). Schematic of synovial tissue and Hfob1.19 cells was shown in Figure 2.

Cell cycle and apoptosis analysis

The cells were trypsinised into a single-cell suspension for cell cycle and apoptosis analysis. After washed by washed twice with PBS (Thermo Fisher Scientific, Inc.) for two times, 10⁶ cells were trypsinised and resuspended in 1 ml PBS. 70% ethanol was blending into cell suspension on ice to form final volume of 4.0 ml. Then cells were incubated with RNAase for 30 min, and 40 µg/ml PI (Yearthbio) was added prior to flow cytometer analysis.

Proliferation assay

Cell Counting Kit-8 (CCK-8) assay was used to evaluate cell viability. Briefly, the hFOB 1.19 cells were collected and seeded into the 96-well plates at a dose of 5×10³/mL. 10 µL of CCK-8 solution (Nanjing Jiancheng Biotechno-
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Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) was used to extract total RNA from hFOB1.19 cells. Target gene and endogenous control β-actin were amplified by qPCR using the SYBR Green PCR Kit (Takara Biotechnology Co., Ltd., Dalian, China). GAPDH was used as an internal reference. The primers for PCR were as follows: GLUT3, forward 5'-CGGCTTCTCATTACCTTC-3', GLUT3, reverse 5'-GGCACGACTTAAGACATGG-3'; CBF-α1, forward 5'-GATGCTCTTAGTGCCCCAAATG-3'; CBF-α1, reverse 5'-GGCTGAAGGTTGACACAG-3', Col-I, forward 5'-GAGGGCCAAAGACGACATC-3'; Col-I, reverse 5'-CAGATCACCTCATGCACACAC-3', OCN, forward 5'-CAGATCACCTCATGCACACAC-3', OCN, reverse 5'-CCTCCTGCTCTTGAAGACACAAAG-3', OPN, forward 5'-CAAATACCCAGATGCTGTCG-3', OPN, reverse 5'-GGCTGTGTTCTCTCACTTCTC-3'. The thermocycling conditions were 25°C for 5 min, 42°C for 60 min and at 95°C for 15 sec. Fold changes were calculated using the 2^ΔΔCq approach normalized to GAPDH.

Western blot analysis

Hfob1.19 cells were harvested on ice in PBS and centrifuged. Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck-Millipore, Darmstadt, Germany) and quantified using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Suzhou, China). 20 µg proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel) and transferred onto polyvinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA). The PVDF membranes were then

Cell transfection

Hfob1.19 cells were transfected with small interfering RNA (siRNA) or c-DNA as previously described [13]. Hfob1.19 cells were placed into 6-well plates for 24 h. Cells were transfected with specific siRNA (Ambion, Huntingdon, UK) or c-DNA targeting JNK and p38 with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 48 h, the cells were co-cultured with a volume of approximately 85 mm^2 of RAS for consistent 7 days. Then, cells were collected for western blot analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

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blocked with 5% nonfat milk for 2 h at room temperature and incubated with GLUT3, core binding factor α1 (CBF-α1), collagen I (Col-I), osteocalcin (OCN), osteopontin (OPN), total c-Jun N-terminal kinase (JNK), p-JNK, p-p38, total p38 antibodies (1:500; Inc., Beverly, MA, USA) and GAPDH antibody (1:3000, Sigma-Aldrich, USA) overnight at 4°C. Then, the membranes were re-probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Inc.). The signal was visualized using an HRP chemiluminescent substrate reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Then, an ECL Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.) was used for immunodetection and densitometry was performed using image J (Version 1.25, Bethesda, MD, USA).

Statistical analysis

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The differences between groups were compared using t-test or one-way ANOVA. P<0.05 was considered statistically significant.

Results

Cell proliferation

Our findings suggested that the cell viability of Hfib1.19 cells was significantly suppressed since the day 3 after co-cultured with RAS (Figure 3A). Furtherly, the mRNA expression and protein expression of GLUT3 were markedly decreased as compared with the Hfib1.19+NS group (Figure 3B, 3C). Flow cytometry detection demonstrated that co-cultured with RAS remarkably suppressed cell cycle when compared with the Hfib1.19+NS group. G0/G1 phase cell gradually increased when co-cultured with RAS, while S phase and G2/M phase cell did not obviously change (Figure 3D). However, Flow cytometry revealed that there was no significant difference between Hfib1.19+NS group and Hfib1.19+RAS group of Hfib1.19 cells. These results all suggested that RAS suppressed the proliferation of hFOB 1.19 cells.

Gene and protein expressions of bone markers

To explore the effect of RAS on the mineralization of osteoblasts, bone markers, such as CBF-α1, Col-I, OCN and OPN were valued via RT-PCR. It revealed that no significant changes have been found at day 7, day 14 and day 28, which suggested that RAS might not decrease the osteogenesis ability of osteoblasts within 28 days (Figure 4). Similar results of the protein expressions were found in Figure 5A, 5B. It suggested that there were no significant differences were found between the two groups on the protein expression of CBF-α1, Col-I, OCN and OPN.

JNK and p38 pathway

To character the mechanism of RAS on osteoblasts proliferation, the effects of JNK and p38 were determined by evaluating signal pathway activity and cell proliferation. Our findings suggested that the protein expressions of p-JNK and p38 were markedly down-regulated after co-cultured with RAS. Little changes of total JNK and total p38 protein expressions were found between the two groups (Figure 5C, 5D). To determine the time variation of JNK and p38 phosphorylation, the protein expressions of p-JNK and p-p38 were valued. It suggested that the protein expression of p-JNK was effectively suppressed since the day 3, and p-p38 was markedly suppressed since the day 7, respectively (Figure 6).
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The cells were further divided into three groups, including the control group, single Hfob1.19-si-JNK or p38-induced knockdown+RAS group and Hfob1.19-cDNA-induced JNK or p38 overexpression+RAS group. It revealed that p-JNK and p-p38 were effectively down-regulated via si-JNK or p38-induced knockdown and up-regulated by cDNA-induced JNK or p38 overexpression (Figure 7).

Additionally, cell viability, GLUT3 gene expression and cell cycle were detected, respectively. It suggested that cell viability was effectively suppressed by si-JNK-induced knockdown and promoted via cDNA-induced JNK overexpression from day 3 (Figure 8A). Similarly, the cell viability was effectively suppressed by si-p38-induced knockdown and promoted via cDNA-induced p38 overexpression from day 5 (Figure 8B). Subsequently, the gene expression of GLUT3 was valued, and it revealed that the mRNA expression of GLUT3 could effectively suppressed by si-JNK or p38-induced knockdown, and reversed by cDNA-induced JNK or p38 overexpression (Figure 8C, 8D).

Besides, cell cycle was also detected, and results revealed that cDNA-induced JNK or p38 overexpression promoted the cell cycles when compared with that of the control group. In contrast, si-JNK or p38-induced knockdown effectively blocked the cell cycles as compared with

Figure 3. Effects of RAS on the cell proliferation of Hfob1.19 cells. A. RAS effectively suppressed the cell viability since day 3 valued by CCK-8 assay. B, C. RAS markedly suppressed the GLUT3 expressions detected via RT-PCR and western blot. D. RAS could significantly inhibit the cell cycle of Hfob1.19 cells detected by flow cytometry. E, F. Apoptosis was detected by flow cytometry, and no significant difference was found between the two groups. All data were expressed as mean ± SD, *P<0.05, **P<0.01, as compared with Hfob1.19+NS group.
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The proliferation of osteoblasts has been assessed in this study. Figure 4 illustrates the effects of RAS on the gene expression of bone markers of HFOB1.19 cells at days 7, 14, and 21. RAS exhibited no significant influence on the mRNA expressions of CBF-α1, Col-I, OCN, and OPN, as detected by RT-PCR. Notably, Figure 5 shows the protein expressions of CBF-α1, Col-I, OCN, and OPN, which were detected by western blot. No significant changes in protein expression were observed. Furthermore, RAS markedly suppressed the protein expressions of p-JNK and p-p38, and had no significant influence on the total JNK and p38 expressions. All data were expressed as mean ± SD, with *P<0.05 compared to HFOB1.19+NS group.

Discussion

Rheumatoid arthritis (RA) is characterized by focal articular erosion that is mediated by regulating the balance between osteoblasts and osteoclasts [14, 15]. Cortical bone around the joints is the initial target of bone erosion following RA [16]. Progression of bone erosion leads to subchondral bone loss and contributes to destruction of articular cartilage [17]. Interdiction of bone erosions in RA patients has been documented by excision of invaded synovial tissue, which to some extent, suggests an ongoing suppression of function of bone-forming osteoblasts [18]. Bone metabolism is regulated at multiple levels by factors that control osteoblast function. Synovial tissue as an organized whole has been reported to have erosive effects on osteoblasts.
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Figure 6. Effects of RAS on the protein expressions of p-JNK and p-p38 of HFOB 1.19 cells at day 0, 3, 7 and 11. The protein expression of p-JNK was effectively suppressed since the day 3, and p-p38 was markedly suppressed since the day 7, respectively. All data were expressed as mean ± SD, *P<0.05, **P<0.01, as compared with day 0 group.
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Figure 7. Effects of RAS on the decreased MAPK phosphorylation of Hfob1.19 cells. siRNA-induced knockdown of (A) JNK and (B) p38 promoted the inhibitory effects of RAS on JNK and p38 phosphorylation. cDNA-induced overexpression of (A) JNK and (B) p38 suppressed the inhibitory effects of RAS on JNK and p38 phosphorylation. All data were expressed as mean ± SD, **P<0.01, as compared with control group.

A  B

Cell viability (OD450nm)

1d  3d  5d  7d

C  D

mRNA expression of GLUT3

Contorl  si-JNK  JNK-cDNA

E  F

Cell ratio (%)

G0/G1  S  G2/M

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Figure 8. Effects of RAS on the cell proliferation of Hfob1.19 cells followed by MAPK knockdown or overexpression. siRNA-induced knockdown of JNK and p38 effectively promoted the inhibitory effects of RAS on (A/B) cell viabilities, (C/D) GLUT3 gene expressions and (E/F) cell cycles. While, cDNA-induced overexpression of JNK and p38 markedly suppressed the inhibitory effects of RAS on (A/B) cell viabilities, (C/D) GLUT3 gene expressions and (E/F) cell cycles. All data were expressed as mean ± SD, *P<0.05, **P<0.01, as compared with control group.

of osteoblasts and matrix mineralization [42]. Thouverey et al. [43] found that the tibial bone mass decreased by 62% for six months in the MAPK p38 knockout mice, which suggested that the MAPK p38 pathway plays an important role in regulating the bone formation of osteoblasts. In addition, previous studies have reported that MAPK p38 are crucial in the maintenance of the cell survival and proliferation of osteoblasts [37, 42].

Based on the characteristics of RA and the important role of the MAPK signaling pathway in osteoblasts, we noticed that JNK and p38 signal transduction when Hfob1.19 cells were co-cultured with RAS. Therefore, we suspected that JNK and p38 might regulate the proliferation of Hfob1.19 cells through MAPK pathway. The cells were further divided into three groups, including the control group, single Hfob1.19-si-JNK or p38 induced knockdown+RAS group and Hfob1.19-cDNA induced JNK or p38 overexpression+RAS group. It suggested that JNK and p38 knockdown could effectively suppress the proliferation and cell cycle of osteoblasts, and JNK and p38 overexpression could significantly reverse this phenomenon, which suggested that RAS suppressed the proliferation of osteoblasts probably via inhibition of JNK and p38 pathway.

The complex bone erosion microenvironment could be formed when osteoblasts co-cultured with rheumatoid arthritis derived synovial tissue. Synovial tissue produces certain molecules, such as exosomes and microRNAs [44, 45]. Additionally, synovial extracellular matrix can release some pro-inflammatory factors, such as TNF-α, IL-1 [44, 46]. These exosomes, microRNAs and pro-inflammatory factors might be released into the culture medium during the co-culture process and have an impact on the JNK and p38 expression of osteoblasts and ultimately induced cells inhibition. The specific mechanisms still need to be further explored. Although no significant changes of bone markers were observed in this experiment, the reasons may be summarized as follows: the co-culture time of RAS and Hfob1.19 cells is not long enough to stimulate osteoblasts sufficiently. The amount of synovial tissue is too small that substances, such as exosomes, microRNAs and pro-inflammatory factors secreted by synovial tissue could not reach the effective concentrations to make the changes of bone markers in osteoblasts.

In conclusion, we preliminarily established a co-culture system of synovial tissue from patients with rheumatoid arthritis and osteoblasts in vitro, and we showed that RAS could suppress the proliferation of osteoblasts via MAPK pathway, which might contribute to exploring the interaction of synovial tissue with bone cells as well as drug sensitivity to guide the treatment of RA.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

RAS, rheumatoid arthritis-derived synovial tissue; NS, normal synovial tissue; Col-I, collagenI; OCN, osteocalcin; OPN, osteopontin; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase.

Address correspondence to: Yuefeng Hao and Dan Hu, Department of Orthopaedics, The Affiliated Suzhou Hospital of Nanjing Medical University, 242 Guangji Road, Suzhou 320500, PR China. Tel: +86 13913109339; E-mail: 13913109339@163.com (YFH); Chicago_Hudan@163.com (DH)

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