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Chemerin affects the metabolic and proliferative capabilities of chondrocytes by increasing the phosphorylation of AKT/ERK

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Abstract. – OBJECTIVE: The purpose of the present study was to explore the mechanism of action of the adipokine chemerin in osteoarthritis (OA) by means of an in vitro OA model.

MATERIALS AND METHODS: Primary chondrocytes were isolated from normal rats. The chondrocytes were stimulated with interleukin 1 beta (IL-1β, 10 μg/L) to establish a model of induced OA. Chemerin was administered to cells of this model. After culture of the chondrocytes in the presence of chemerin for 48 h, the expression of the genes related to OA occurrence and protection, matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3), and matrix metalloproteinase-13 (MMP-13) was examined. Western blot was then performed to analyze the phosphorylation of the AKT and extracellular signal-regulated kinase (ERK) proteins in chondrocytes.

RESULTS: Stimulation of chondrocytes with IL-1β markedly reduced the proliferative capability of chondrocytes. Chemerin (5 μM) also significantly decreased the proliferative capability of chondrocytes. The combined administration of IL-1β and chemerin induced an even greater reduction in the proliferative capability of chondrocytes. Polymerase chain reaction (PCR) results showed that both IL-1β and chemerin reduced the expression of the protective genes in OA (MMP-1, MMP-3, and MMP-13). Also, the stimulation with IL-1β and chemerin significantly enhanced the phosphorylation of AKT/ERK in chondrocytes.

CONCLUSIONS: This adipokine induces changes in the metabolic and proliferative capabilities of chondrocytes by increasing the phosphorylation of AKT/ERK, thereby inducing OA or aggravating the symptoms of OA.

Key Words:
Chemerin, Chondrocytes, Osteoarthritis, AKT/ERK.

Introduction

Osteoarthritis (OA) is a type of chronic joint disease. The clinical features of OA include the degeneration, destruction, and hyperostosis of articular cartilage and subchondral bones and synovitis[1]. A variety of factors affect the occurrence of OA. Obesity, trauma, age, and even gender affect the pathogenesis of OA. A number of studies have found that obesity seriously affects the occurrence of knee arthritis and interphalangeal joint arthritis[2]. Adipokines are a class of endogenous bioactive polypeptides secreted by white adipose tissue. Adipokines are closely related to a variety of metabolic disorders, such as obesity, insulin resistance, and diabetes. Our previous study[3] found that chemerin, an adipokine in synovial fluid, was positively correlated with the severity of knee OA. Therefore, we established an *in vitro* model of inflammation using rat articular chondrocytes and investigated the effects of chemerin on degenerative chondrocytes on the basis of the model. In addition, we explored the mechanisms of action of the adipokine chemerin at the cellular level.

Materials and Methods

Animals

A total of 3 male Wistar rats were used in the present study. The rats were 4-6 weeks old and purchased from Vital River Laboratory Animal Technology Co., Ltd. (Vital River, Beijing, China).

Reagents

Dulbecco's modified Eagle medium (DMEM), D-Hanks solution and fetal bovine serum (FBS) were purchased from Gibco Life Sciences (Gibco, Grand Island, NY, USA). Trypsin and collagenase were purchased from Thermo Fisher Scientific Inc (Thermo Fisher Scientific, Waltham, MA, USA). Interleukin 1 beta (IL-1β) and chemerin were purchased from Sigma-Aldrich Corporation (Sigma-Aldrich, St. Louis, MO, USA). Pri-

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mary antibodies (anti-AKT/p-AKT, anti-extracellular signal-regulated kinase (ERK)/p-ERK, anti-chemerin chemokine-like receptor 1 (CMKLR1), and anti-GAPDH antibodies) and secondary antibodies (rabbit anti-mouse and mouse anti-rabbit IgG) were purchased from Abcam (Cambridge, MA, USA).

Materials and Equipment

The 100-ml Petri dishes, 15-ml centrifuge tubes, 24-well plates and 96-well plates were all purchased from Corning Inc. (Corning, NY, USA). The CO_2 incubator and laminar flow clean benches were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA). The inverted microscope was purchased from Olympus Corporation (Olympus, Tokyo, Japan). The fully automatic full-wavelength microplate reader was purchased from Life Technologies Corporation (Life Technologies, Waltham, MA, USA).

Isolation and Culture of Rat Chondrocytes

Articular cartilage was collected from the knee joints of Wistar rats in a laminar flow cabinet. The articular cartilage was minced into small pieces approximately 1 mm^3 in size. Subsequently, the articular cartilage fragments were digested with trypsin and type II collagenase to obtain a cell suspension. After termination of the digestion, the cells were centrifuged, resuspended in DMEM containing 10% FBS, seeded into tissue culture dishes and placed at 37°C in a 5% CO_2 incubator. The culture medium was replaced at 12 h after the cells had adhered to the culture surface. Once the cells reached 80% confluence, they were rinsed once with phosphate-buffered saline (PBS) and trypsinized. The chondrocytes were collected by centrifugation and then passaged.

Cell Proliferation Assay

Chondrocytes that were in the logarithmic growth phase were collected and then seeded into 96-well plates at a density of 2.5×10^4 cells/ml (200 µl per well). At 12 h after the cells became adherent to the culture surface, the culture medium was replaced with medium containing various concentrations of chemerin (0, 1, 5, 10, and 20 µM) (Sigma-Aldrich, St. Louis, MO, USA). Chondrocytes were cultured for 48 h in the presence of chemerin. Subsequently, 10 µl of Cell Counting Kit-8 (CCK-8) solution (Beyotime Biotechnology, Shanghai, China) was added to each well of cells. After incubation of the cells for 4 h at 37°C in a 5% CO_2 incubator, the absorbance at 450 nm was measured using a microplate reader. The growth curves were plotted, and the effects of various concentrations of chemerin on chondrocyte proliferation were compared. A model of chondrocyte inflammation was established by stimulation of chondrocytes with IL-1β (10 µg/L) for 48 h. The proliferation of chondrocytes was examined using the method described above. Chondrocytes were stimulated with the adipokine chemerin. Specifically, cultured chondrocytes were divided into 4 groups: the control (ctrl) group, the chemerin group, the IL-1β group, and the IL-1β+chemerin group. Chemerin was used at a concertation of 5 µM, while the concentration of IL-1β was 10 µg/L. The proliferation of the 4 groups of chondrocytes was examined using the method described above.

Examination of the Expression of the OA-Related Genes by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Chondrocytes were cultured *in vitro* until passage 3. The cells were then divided into the following 4 groups: the ctrl group, the chemerin group, the IL-1β group, and the IL-1β+chemerin group. Chemerin was used at a concentration of 5 µM, while IL-1β was used at a concentration of 10 µg/L. Once the cells adhered to the culture surface, they were subjected to the drug stimulation corresponding to their group. After 48 h of stimulation, RNA was collected. Total RNA (Qiagen, Hilden, Germany) was extracted using the TRIzol method, quantified and reverse transcribed into complementary DNA (cDNA). Subsequently, fluorescence-based quantitative PCR (Applied Biosystems, Foster City, CA, USA) was performed. The indices examined by PCR included glyceraldehyde 3-phosphate dehydrogenase (GAPDH), matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-3 (MMP-3), and matrix metalloproteinase-13 (MMP-13). The primer sequences are shown in Table I.

Western Blot (WB) Analysis of the Expression of the Phosphorylated Forms of the Inflammatory Proteins AKT and ERK

Chondrocytes were cultured *in vitro* until passage 3. The cells were then divided into the following 4 groups: the ctrl group, the chemerin group, the IL-1β group, and the IL-1β+chemerin group. Chemerin was used at a concentration of

Table I. Primer sequences used in PCR-based gene expression analysis.

Gene	Primer sequence (3′-5′)
GAPDH	GGAGAAAGCTGCTAA
	ACGACCTGGTCCTCGGTGTA
MMP-1	GTGTGGAGTGCCTGATGTG
	CTGCTTGACCCTTGGAGAC
MMP-3	TTCCAACCCTGCTACTGC
	TGCCTGTCACCTCCAAG
MMP-13	CTTCTGGCTCACGCTTTTC
	TTGGGGTCTTCATCTCCTG

5 μM, while IL-1β was used at a concentration of 10 μg/L. Once the cells adhered to the culture surface, they were subjected to the drug stimulation corresponding to their group. After 48 h of stimulation, proteins were collected. First, the culture medium was discarded. The cells were then washed twice with PBS, overlaid with an appropriate amount of radioimmunoprecipitation assay (RIPA) lysis buffer and lysed on ice for 30 min. The concentrations of the proteins in the cell lysates were measured using the bicinchoninic acid (BCA) assay (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was adjusted to a uniform concentration. Subsequently, the proteins were denatured at 95°C for 10 min, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich, St. Louis, MO, USA). The membranes were blocked with 5% skim milk for 1 h and then incubated with primary antibodies (anti-CMKLR1, anti-p-AKT/AKT, and anti-p-ERK/ERK antibodies, 1:1000 dilution) at 4°C overnight. After being washed 3 times, the membranes were incubated with the secondary antibodies for 2 h. Following incubation with the secondary antibodies, the membranes were washed 3 times. The protein bands were then visualized.

Results

Cell Morphology and Identification

The chondrocytes obtained by collagenase digestion displayed an irregular polygonal or oblate morphology. After 2-3 passages, the cells became polygon shaped (Figure 1).

Proliferative Activity of Chondrocytes

The chondrocytes were treated with various concentrations of chemerin for 48 h. Subsequently, a CCK-8 assay was conducted to examine the proliferation of the 2 groups of chondrocytes and compare the proliferative activity of the chondrocytes. As shown in Figure 2A, 5 μM chemerin markedly suppressed the proliferation of chondrocytes. Figure 2B shows that 10 μg/L IL-1β significantly reduced the proliferative capability of chondrocytes. The combined administration of chemerin and IL-1β resulted in an even more noticeable decline in the proliferative capability of chondrocytes. The results demonstrate that chemerin alone is sufficient to induce changes in the metabolic and proliferative capability of chondrocytes. The combined application of chemerin and IL-1β will reduce the proliferative capability of chondrocytes to a greater extent.

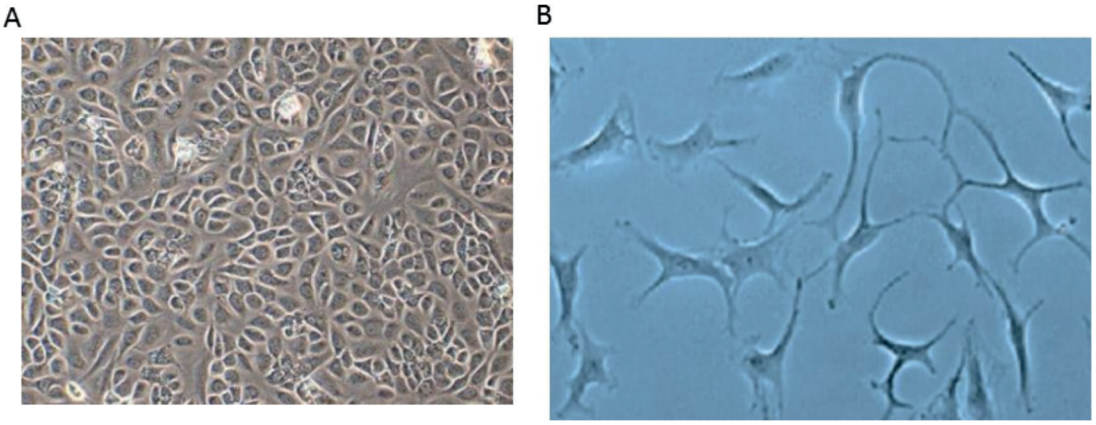

Figure 1. Morphology of chondrocytes. ***A,*** The newly isolated chondrocytes mainly displayed an oblate morphology. ***B,*** The chondrocytes cultured for 3 days mainly adopted a polygonal morphology.

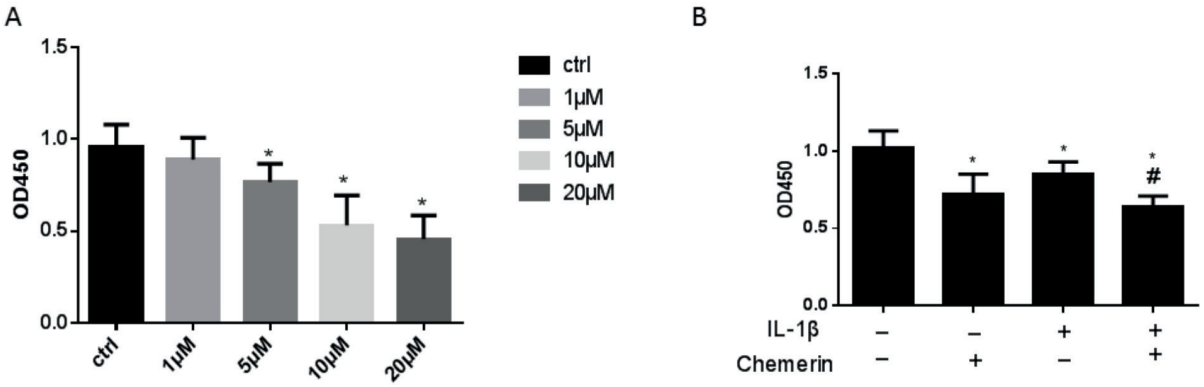

Figure 2. Effect of the adipokine chemerin on the proliferation of chondrocytes. ***A,*** Effects of various concentrations of chemerin on the proliferative capability of normal chondrocytes. ***B,*** Effect of chemerin on the proliferative capability of the chondrocytes with inflammation induced by IL-1β stimulation. *indicates $p < 0.05$ compared with the ctrl group. #indicates $p < 0.05$ compared with the IL-1β group.

RNA Expression Levels of OA-Related Genes

To examine the effects of different stimulus factors on the expression of the OA-related cellular factors (MMP-1, MMP-3, and MMP-13) in chondrocytes, qRT-PCR was performed. Stimulation of chondrocytes with IL-1β increased the expression of the OA-related genes in chondrocytes, indicating that IL-1β significantly affected the structure of chondrocytes and induced changes in the cytoskeleton of chondrocytes. Chemerin alone was capable of altering the cytoskeletal structure of chondrocytes. The combined administration of IL-1β and chemerin would aggravate the changes.

Examination of the Protein Expression of Inflammatory Factors in Chondrocytes by WB

WB analysis of the expression of OA-related proteins in chondrocytes showed that stimulation with chemerin and IL-1β significantly increased the expression of the chemerin receptor CMKLR1. The expression of p-AKT and p-ERK was also elevated. The results indicate that chemerin activates p-AKT and p-ERK by activating its receptor CMKLR1, thereby promoting the expression of inflammatory factors in chondrocytes.

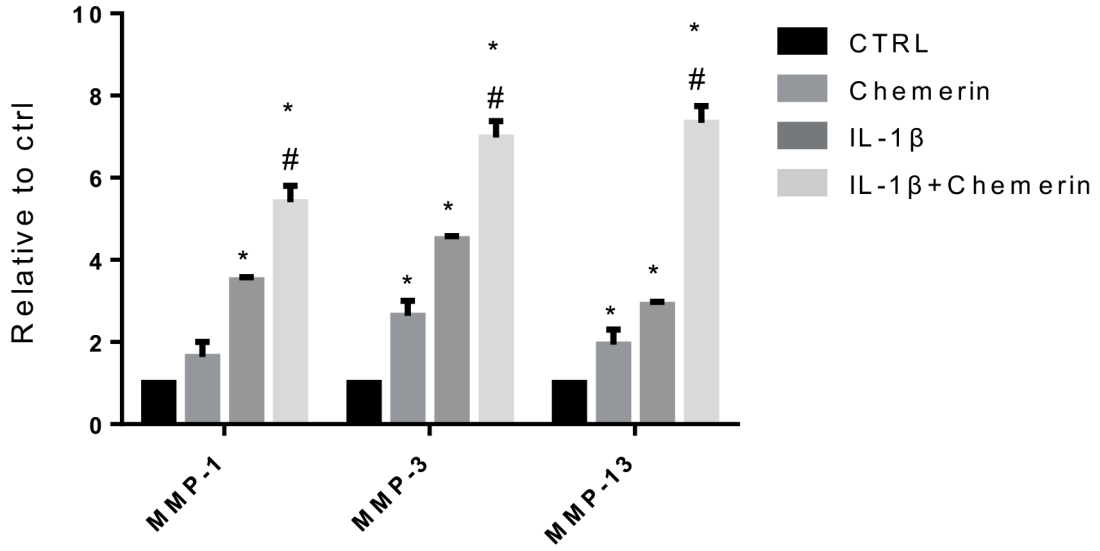

Figure 3. Examination of the expression of OA-related genes in chondrocytes by qRT-PCR. *indicates $p < 0.05$ compared with the ctrl group. #indicates $p < 0.05$ compared with the IL-1β group.

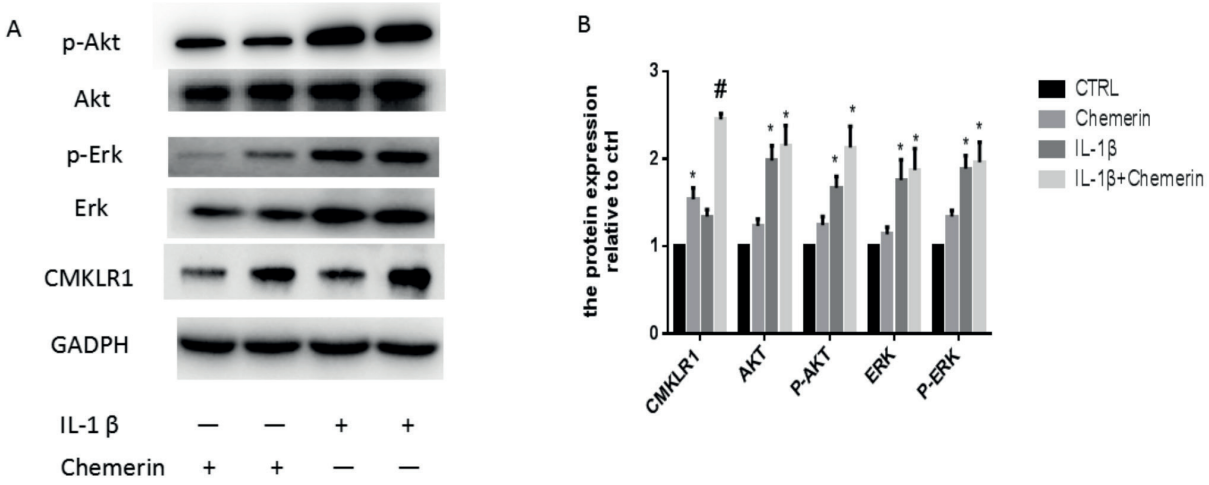

Figure 4. WB analysis of the expression of OA-related cytokines in chondrocytes. ***A,*** Results of WB analysis. ***B,*** Statistical analysis of the results from Figure 4A. *indicates $p < 0.05$ compared with the ctrl group. #indicates $p < 0.05$ compared with the IL-1β group.

Discussion

Chemerin is an adipokine that was first identified in 2007. It consists of 163 amino acid residues and is secreted in the form of a pro-protein. Chemerin becomes activated and exhibits its biological activity after being hydrolyzed by cysteine protease or serine protease[4]. Eisinger et al[5] reported that chemerin stimulates the migration of leukocytes to joints, thereby enhancing the severity of OA. The study also showed that synovial fibroblasts release chemerin. This factor further regulates the expression of toll-like receptor 4 (TLR4) and the release of chemokine (C-C motif) ligand 2 (CCL2) in synovial fibroblasts through a feedback mechanism. The results demonstrate that chemerin plays an important role in OA.

Habib et al[6] reported that serum chemerin levels are elevated type 2 diabetes patients. Also, chondrocytes are the principal cellular constituent of articular cartilage. The main role of chondrocytes is to maintain the morphology and normal function of cartilage. Chondrocyte senescence and apoptosis are the main causes of OA. However, the molecular mechanisms underlying the morphological changes and dysfunction of chondrocytes remain unclear at present. A variety of cytokines are abnormally expressed during the pathogenesis of OA, resulting in disordered metabolism and function of chondrocytes. Members of the matrix metalloproteinase (MMP) family are widely present in various connective tissues. MMPs play important roles in the metabolism of cellular matrices, including the metabolism and degradation of the chondrocyte matrix in OA[7]. A large number of studies showed that increased expression of MMPs significantly promotes extracellular matrix degradation. Therefore, an increase in the expression of the members of the MMP family in chondrocytes indicates changes in the metabolic and proliferative capability of the chondrocytes. IL-1 plays an important role in OA[8]. A number of studies[9,10] reported the successful simulation of OA *in vitro* by application of IL-1β to induce inflammation in chondrocytes. The present work first verified whether the model of IL-1β-induced chondrocyte inflammation could be successfully established. We found that the expression of MMPs was significantly elevated, and the proliferative capability was decreased in chondrocytes exposed to the inducer. Our results agreed with the findings reported previously.

In the present study, chondrocytes were stimulated with the adipokine chemerin, and IL-1β was administered to induce inflammatory responses in chondrocytes. Chemerin not only affected the proliferative capability of normal chondrocytes but also aggravated the inflammatory responses of chondrocytes. In OA, AKT is phosphorylated and activated in chondrocytes, which is achieved mainly by the activation of sirtuin 1 (SIRT1). SIRT1 activation affects the expression of phosphoinositide 3-kinase (PI3K), thus promoting changes in the phosphorylation status of AKT[11]. In addition, AKT phosphorylation significantly promotes the expression of MMP-13 by chondrocytes[12]. The levels of phosphorylated c-Jun N-terminal kinases (p-JNKs), p38, and ERK are significantly higher in the

cartilage of OA patients than in normal cartilage[13,14]. The finding indicates that the mitogen-activated protein kinase (MAPK) signaling pathways consisting of the ERK, JNK, and p38 proteins also play important roles in the pathogenesis of OA and that the expression of MMPs in chondrocytes is related to the activation of MAPK[15,16]. The above results demonstrate that the activation of the MAPK signaling pathway and the activation of the PI3K signaling pathway affect the expression of the MMP family of proteases, which further affects the function of chondrocytes. In the present work, WB analysis showed that chemerin promoted the expression of its receptor CMKLR1 and the phosphorylation of AKT and ERK in chondrocytes. These findings indicate that chemerin not only regulates the expression of inflammatory factors by controlling the phosphorylation of AKT/ERK proteins but also modulates the expression of the MMP family of signaling factors in chondrocytes, thereby affecting the metabolic and proliferative capability of chondrocytes and promoting the occurrence of OA.

Conclusions

We observed the effect of chemerin on the proliferative capability of chondrocytes and explored the potential mechanisms of action of chemerin. Chemerin induced inflammatory responses in chondrocytes and reduced the proliferative capability of chondrocytes, thereby regulating the severity of OA. In this report we provide a theoretical basis for the application of chemerin as a biomarker of OA.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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