International Immunopharmacology 60 (2018) 228–234

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

IL-9 exhibits elevated expression in osteonecrosis of femoral head patients and promotes cartilage degradation through activation of JAK-STAT signaling in vitro

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ARTICLE INFO

Keywords:
Interleukin-9
JAK-STAT
Inflammation
Osteonecrosis of the femoral head
TNF-α

ABSTRACT

Osteonecrosis of the femoral head (ONFH) often causes severe symptoms in young people and limits the mobility of the hip joint. Interleukin-9 (IL-9) is a multi-functional inflammatory factor that participates in lumbar disk herniation and arthritis and has been reported in many studies. However, the correlation between IL-9 and ONFH is unclear. The present study aimed to determine the role of IL-9 in the pathogenetic mechanism of osteonecrosis. To assess IL-9 expression in ONFH and femoral neck fracture patients, cartilage tissue was examined through western blot analysis and immunohistochemistry. Human primary chondrocytes were stimulated with IL-9, and inflammation-related cytokines and cartilage matrix-degrading enzymes were assessed via real-time PCR. After being treated with IL-9, Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling were tested through western blot analysis. Our results showed a significant increase in the expression of IL-9 in ONFH patients. IL-9 raised the level of inflammation-related cytokines and cartilage matrix-degrading enzymes and enhanced the activation of JAK-STAT signaling. Furthermore, blocking the JAK-STAT signaling pathway reduced the secretion of inflammation-related cytokines and cartilage matrix-degrading enzymes and markedly alleviated the degradation of the cartilage matrix. These findings provide new insights into the role that IL-9 plays in the pathogenetic mechanism of osteonecrosis and also provide a potential treatment for ONFH.

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a common disease in middle age [1]. Patients suffer from pain and limited joint motion, which affect their work and quality of life. The hip joint destruction caused by ONFH is irreversible, and there is no cure. Many studies have reported that inflammation is a critical factor involving the destruction of cartilage. However, the underlying mechanism of ONFH remains unresolved.

Interleukin-9 (IL-9), initially known as P40, is a 14KD soluble glycoprotein [2]. The human IL-9 gene, located on chromosome 5 [3], is in the Th2 cytokine gene cluster [4]. It is produced by many types of cells, including T helper cells (Th2 cells) [5], Th9 cells [6], Th17 cells [6], natural killer cell (NKT cells) [7], mast cells [8], regulatory T cells (Treg cells) [9] and group 2 innate lymphoid cell (ILC2 cells) [10]. IL-9 plays a critical role in inflammatory diseases, such as asthma [11], atopic dermatitis [12], lumbar disc herniation [13], systemic lupus erythematosus [14], systemic sclerosis [15] and rheumatoid arthritis [16]. It has been demonstrated that playing a pro-inflammatory or anti-inflammatory role depends on the source of the IL-9 secretion and the stage of the disease [17]. The effect of IL-9 on cartilage degeneration in ONFH patients is unknown. Herein, we assessed the expression of IL-9 in the articular cartilage and blood of ONFH patients and investigated the role of IL-9 on cartilage degeneration in an in vitro human primary chondrocyte culture model.

2. Materials and methods

2.1. Patients, specimen source and ethics statement

Human blood samples were obtained from healthy individuals ($n = 20$, age 23–45 years) and patients with ONFH ($n = 20$, age 35–60 years). Femoral head cartilage specimens were obtained from femoral neck fracture (FNF) patients without ONFH and osteoarthritis ($n = 20$, age 43–65 years) and ONFH patients. All samples were collected between November 2016 and August 2017 in Liaocheng City People's Hospital, Liaocheng, China. In the ONFH group, all patients were classified as having stage III or IV ONFH, based on the Ficat

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https://doi.org/10.1016/j.intimp.2018.05.005
Received 17 March 2018; Received in revised form 1 May 2018; Accepted 9 May 2018
Available online 25 May 2018
1567-5769/

classification [18]. The following inclusion criteria were applied: An accurate diagnosis of ONFH and FNF of all enrolled patients was confirmed through the examination of hip X-rays and magnetic resonance imaging (MRI). The following exclusion criteria were applied: patients with primary autoimmune disorders, such as osteoarthritis, spondylolysis, spondylolisthesis, rheumatoid arthritis, systemic lupus erythematosus, or other immunological disorders, and patients with recent use of glucocorticoid and a history of tumor. The present study was approved by the Medical Ethical Committee of Liaocheng City People's Hospital, Taishan Medical College. Informed consent was obtained from all participants involved in this research.

2.2. Immunohistochemistry (IHC)

Cartilage tissue from each patient was collected and fixed in 10% formalin, decalcified, dehydrated, and cleared with dimethylbenzene, and then embedded in paraffin to prepare at least 5-μm thick sections. After being deparaffinized and rehydrated, as well as inhibiting endogenous peroxidase activity and blocking off the nonspecific protein binding sites, the serial paraffin sections were incubated with primary antibodies, including rabbit anti-human IL-9 (1:500 dilution; Abcam, USA), rabbit anti-tumor necrosis factor-α (TNF-α) (1:300 dilution; Abcam), rabbit anti-matrix metalloproteinase-13 (MMP-13) (1:300 dilution; Abcam), rabbit anti-IL-6 (1:400 dilution; Abcam), rabbit anti-phosphorylated-STAT3(p-STAT3) (1:200 dilution; Abcam), rabbit anti-p-STAT1 (1:300 dilution; Abcam). The samples were stored overnight at 4 °C, followed by a secondary antibody incubation (goat anti-rabbit immunoglobulin (IgG)-horseradish peroxidase HRP; 1:500 dilution; ZSGB-Bio, China) at 37 °C for 30 min. Images were captured with an IX71-SIF type microscope (Olympus, Tokyo, Japan) at 200× magnification and analyzed using ImagePro Plus 5.0 software (Media Cybernetics, MD, USA).

2.3. Safranin O staining

The paraffin sections were prepared using IHC, as previously described. After being deparaffinized and hydrated, paraffin sections from the two groups were successively stained with hematoxylin for 2 min, washed with clean water for 15 min, stained with 0.001% fast green for 5 min, rinsed in acetic acid solution for 3 s and stained with 0.1% safranin for 20 min. Finally, after being dehydrated with 95% and 100% hexanol for 2 min and transparentized with dimethylbenzene, the samples were sealed with neutral resin and then examined under a microscope.

2.4. Isolation, culture and stimulation of primary chondrocytes

Human cartilage samples were obtained from our enrolled FNF patients who underwent total hip joint replacement surgery. Primary articular cartilage cells were isolated from the articular cartilage through enzymatic digestion and cultured, as previously reported [19]. Briefly, cartilage tissue was washed with 1% PBS and minced finely into approximately 1 mm^3 pieces. After being digested with 0.25% trypsin and 0.2% type II collagenase (Sigma, USA), the isolated chondrocytes were seeded as a monolayer and cultured in DMEM/F12 media (HyClone, USA) containing 10% FBS (HyClone, USA) and 0.1% penicillin-streptomycin. The primary cartilage cells from generation 2 or 3 were harvested and seeded into 6-well plates and then stimulated with 0, 5 and 20 ng/mL IL-9 (R & D Systems, USA). The chondrocytes and supernatant were collected for further analysis.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The serum samples were separated and retained from the FNF and ONFH patients. The IL-9 levels in the serum were measured with human IL-9 ELISA kits (Abcam, USA), following the manufacturer's instructions. In addition, we measured the concentration of TNF-α, prostaglandin E2 (PGE2) and IL-6 in the primary chondrocyte supernatant stimulated by IL-9 using corresponding ELISA kits (Abcam, USA), according to the manufacturer's protocol.

Table 1
RT-PCR primers.

Target	Forward primer, 5′-3′	Reverse Primer, 5′-3′
ADAMTS-7	GCAGGTTGAGAGCTATGTGCT	GCATGGTGCGTGATCTTTAGG
ADAMTS-4	GGCTAAAGCGCTACCTGCTA	GAGTCACCACCAAGCTGACA
Aggrecan	GGCACTAGTCAACCCTTTGG	CTGAACCCTGGTAACCCTGA
COL2A1	GTGAGCCATGATTCGCCTCGG	CACCAGGTTCACCAGGATTGCC
COMP	TTTTGAATTCGCGACACTGAC	GTCCCGAGAGTCCGT ATGTC
COX-2	GGAACTTTCTGGTCCCTTCAG	TGTGTTTGGAGTGGGTTTCA
IL-6	CAATGAGGAGACTTGCCTGG	GCACAGCTCTGGCTTGTTCC
MMP-13	TGCTGCATTCTCCTTCAGGA	ATGCATCCAGGGGTCCTGGC
TNFα	TGAAAGCATGATCCGGGACG	TGAGGTACAGGCCCTCTGAT
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG

2.6. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from the articular cartilage or cultured primary chondrocyte of each experimental group using the RNeasy kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. The first-strand cDNA was established through a commercial RT-PCR kit (Toyobo, Japan). RT-PCR was performed with SYBR Green I dye to monitor tor DNA synthesis. Data from each sample were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then analyzed using Light-Cycler analysis software 4.0.0.23 (Roche, Switzerland). The nucleotide sequences of TNF-α, IL-6, cyclooxygenase-2 (COX-2), a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4), ADAMTS-7, MMP-13, cartilage oligomeric matrix protein (COMP), type II collagen (COL2A1), aggrecan and GADPH primers used for RT-PCR are listed in Table 1. The presence of a single specific PCR product was verified through melting curve analysis for each gene, and every experiment was run in triplicate. The relative expression level of target genes in each group was calculated using the $2-\triangle\triangle Ct$ method.

2.7. Western blot

Total proteins were extracted from articular cartilage or cultured primary chondrocytes of each experimental group, as previously reported; then, the protein concentrations were quantitated using a BCA Protein Assay Kit (Beyotime Biotechnology Co., Beijing, China), according to the manufacturer's instructions. Equal amounts of protein (10 mg) were loaded onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF, Millipore, USA) membranes. After being blocked in 5% nonfat dry milk in Tris-buffered saline Tween 20 (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween 20) for 3 h, the PVDF membranes were incubated with relevant primary antibodies overnight at 4 °C. The primary antibodies used in this study were listed as follows: rabbit anti-ADAMTS-4 (1:2000 dilution, Abcam); anti-ADAMTS-7 (1:2000 dilution, Abcam); mouse anti-COX2 (1:1000 dilution, Abcam); rabbit anti-MMP-13 (1:1500 dilution, Abcam); rabbit anti-STAT-1 (1:2000 dilution; CST, USA); rabbit anti-STAT-3 (1:1000 dilution, CST); rabbit anti-p-STAT-1 (1:1500 dilution, CST); rabbit anti-p-STAT-3 (1:2000 dilution, CST) and mouse anti-GAPDH antibody (1:1000 dilution; Santa Cruz Biotechnology, USA), which served as the loading control. The protein bands were visualized using a FluorChem imaging system (Amersham Imager 600, General Electric Company, USA), and the data analysis was quantified by using ImageJ software (National Institutes of Health, USA).