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Combination of 4-hydroperoxy cyclophosphamide and methotrexate inhibits IL-6/sIL-6R-induced RANKL expression in fibroblast-like synoviocytes *via* suppression of the JAK2/STAT3 and p38MAPK signaling pathway

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ABSTRACT

Although conventional combination therapy is effective for most patients with rheumatoid arthritis (RA), many still do not respond to current therapies. Therefore, novel combination regimens that better target cellular processes involved in RA pathogenesis are required. Preliminary studies have demonstrated the beneficial effects of a combination of cyclophosphamide (CTX) and methotrexate (MTX) in models of RA. Using western blotting, real-time polymerase chain reaction, enzyme-linked immunosorbent assays, and immunofluorescent staining, we demonstrated that the combination of 4-hydroperoxy CTX (4-H-CTX) and MTX inhibited the expression of receptor activator of nuclear factor-κB ligand (RANKL) in fibroblast-like synoviocytes (FLS) treated with the interleukin (IL)-6/soluble IL-6 receptor (sIL-6R) complex. To elucidate the mechanisms underlying this effect, we treated RA-FLS with the JAK2/STAT3 inhibitor AG490 or p38MAPK inhibitor SB203580. The results showed that IL-6/sIL-6R-induced RANKL upregulation required phosphorylation-mediated activation of STAT3 and p38 signaling, and that 4-H-CTX and/or MTX inhibited RANKL expression in IL-6/sIL-6R-stimulated FLS by suppressing JAK2/STAT3 and p38MAPK signaling. This study demonstrated for the first time the inhibitory effects of 4-H-CTX and MTX on RANKL expression in IL-6/sIL-6R-stimulated FLS *via* suppression of STAT3 and p38MAPK phosphorylation. These results identify promising therapeutic agents that might have clinical applications in patients with RA who are at high risk of bone erosion or do not respond well to conventional therapy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent polyarticular synovitis and synovial hyperplasia, eventually resulting in the irreversible destruction of cartilage and bone, as well as disability in the absence of adequate treatment. Receptor activator of nuclear factor-κB ligand (RANKL), a transmembrane protein of the tumor necrosis factor (TNF) superfamily, plays a pivotal role in bone destruction at inflamed joints in RA [1]. Fibroblast-like synoviocytes (FLS), which are stimulated by interleukin (IL)-6, TNF-α, and IL-17, can produce RANKL in the inflamed joints of patients with RA [1,2]. RANKL exerts its functions by binding to its unique receptor RANK. Mice deficient in RANKL are protected from bone erosion in a serum transfer model of arthritis [3]. These observations indicate that RANKL, mainly produced by FLS, plays an important role in bone resorption and loss in RA.

Early diagnosis of RA and prompt initiation of disease-modifying anti-rheumatic drug (DMARD) therapy are key factors for preventing or minimizing RA-associated damage. Compared to monotherapy, conventional combination therapy in RA has long-term radiographic benefits [4]. MTX is a classic DMARD. Preliminary studies have suggested that the cell cycle-nonspecific drug cyclophosphamide (CTX), in combination with the cell cycle-specific drug methotrexate (MTX), shows synergistic effects in a rat model of RA, significantly inhibiting the maturation of dendritic cells (DCs), downregulating the antigen-presenting capacity in DCs, restoring healthy balance between Th17 cells

Abbreviations: 4-H-CTX, 4-hydroperoxy cyclophosphamide; MTX, methotrexate; CTX, cyclophosphamide; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; RANKL, receptor activator of nuclear factor-κB ligand; JAK2, Janus activated kinase 2; STAT3, signal transducer and activator of transcription 3; p38MAPK, p38 mitogen-activated protein kinases

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and regulatory T cells, reducing the levels of inflammatory cytokines, and suppressing the expression of focal adhesion kinases [5–7].

FLS play important roles in synovial pathology as they are major sources of RANKL in patients with RA [1,2]. Therefore, decreasing or blocking RANKL expression in FLS may be crucial for regulating osteoclast differentiation and preventing bone erosion in RA. In the present study, we investigated the effect of 4-hydroperoxy cyclophosphamide (4-H-CTX), an active metabolite of CTX, in combination with MTX on the expression of RANKL in an inflammatory context using cultured human RA-FLS stimulated with IL-6/sIL-6R, and the possible mechanism of the associated signal transduction pathway.

2. Materials and methods

2.1. Cell culture and reagents

Synovial tissues were obtained from patients with active RA who were undergoing knee replacement surgery. All patients fulfilled the American College of Rheumatology/European League Against Rheumatism 2010 criteria for RA [8]. Synovial tissues were harvested and incubated with collagenase type I (1 mg/ml; Gibco, Life Technologies Corporation, Grand Island, NY, USA) for 4–6 h at 37 °C. After digestion, RA-FLS were washed extensively and cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco). After overnight culture, nonadherent cells were removed, and adherent cells were re-cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere. Subculturing was performed when cells reached 80 − 90% confluence. Cells from passages four to six were used in subsequent experiments.

The study protocol was approved by the Ethics Committee of Shanxi Medical University (approval no. 2015LL011). Written informed consent was obtained from all patients.

2.2. Identification of FLS from patients with RA

RA-FLS were identified and characterized by flow cytometry and microscopy. FLS from the rheumatoid synovium express certain markers, but none of them are entirely unique for activated FLS. Cadherin-11 is one of the relatively specific surface markers for RA-FLS [9–11], and it plays a key role in the acquisition of the invasive behavior of activated FLS. In contrast, CD14 and HLA class II are markers expressed by macrophage-like synoviocytes [10]. Therefore, we selected cadherin-11 as the positive surface marker, and CD14 and HLA class II as negative surface markers for RA-FLS.

Cultured FLS were stained with primary anti-cadherin-11 (Cell Signaling, Massachusetts, USA) for 1 h at 4 °C, followed by FITC-conjugated secondary antibody (Sigma, St. Louis, MO, USA) for 1 h at 4 °C. The negative control was prepared by parallel staining of samples with IgG1 isotype-FITC (BD Biosciences, San Jose, CA, USA). After incubation, cells were rinsed with phosphate-buffered saline and analyzed on a BD FACSCalibur flow cytometer (BD Biosciences). APC-conjugated anti-CD14 monoclonal antibody (mAb) (BD Biosciences) and FITC-conjugated anti-HLA class II mAb (BD Biosciences) were also used for FACS analysis. The negative controls were prepared by simultaneously staining samples with IgG2a isotype-APC (BD Biosciences) or IgG2a isotype-FITC (BD Biosciences). The cell concentration was adjusted to 5×10^5/ml, and the cells were incubated with mAbs for 1 h at 4 °C, rinsed with phosphate-buffered saline, and then analyzed using a flow cytometer. FLS morphology was analyzed using an Olympus IX71 microscope and images were obtained using an Olympus digital camera (Tokyo, Japan).

2.3. 4-H-CTX and MTX treatment

RA-FLS (1×10^5/ml) were cultured in six-well plates, stimulated with IL-6 (PeproTech, Rocky Hill, NJ, USA)/sIL-6R (PeproTech) for 1 h, and then incubated for 72 h in the presence or absence of 4-H-CTX (1 μg/ml; TRC, North York, ON, Canada) or MTX (100 nM; Sigma, St. Louis, MO, USA). FLS not treated with IL-6/sIL-6R were used as control. Recombinant human IL-6 and sIL-6R (100 ng/ml) were used in all experiments.

2.4. Cell viability assay

RA-FLS cells were seeded at 8×10^4/ml in 96-well cell culture flat-bottom plates. Then, FLS were subjected to the treatments described above. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma) according to manufacturer's instructions. MTT (20 μl, 5 mg/ml) solution was added to each well. After incubation at 37 °C for 4 h, the MTT solution was removed and 150 μl of dimethyl sulfoxide was added. The cells were incubated for another 10 min at 37 °C. Finally, the absorbance was read at 490 nm using a microplate reader (Wellscan MK3; Labsystems Dragon, Vantaa, Finland).

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To determine the appropriate concentration of IL-6/sIL-6R and the suitable incubation time, RA-FLS (1×10^5/ml) were cultured in six-well plates and treated with 0, 10, 25, 50, 100, and 200 ng/ml IL-6 (PeproTech)/sIL-6R (PeproTech) for 1 h, or RA-FLS were stimulated with 100 ng/ml IL-6/sIL-6R for various incubation times (0, 6, 24, 48, 72, and 96 h). The cells were harvested, and total RNA was extracted using the E.Z.N.A. total RNA kit (Omega, Irving, TX, USA). Precipitated RNA was reverse transcribed using the PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). The mRNA levels of β-actin and target genes were determined by qRT-PCR using SYBR Green qPCR master mix (TaKaRa). qRT-PCR was performed using specific primers. *RANKL* primer sequences were as follows: forward, 5′-ACCAGCATCAAAATCC CAAG-3′ and reverse, 5′-CCCCAAAGTATGTTGCATCC-3′. Osteoprotegerin (*OPG*) primer sequences were as follows: forward, 5′-TTCAGGTTTGCTGTTCCTACAA-3′ and reverse, 5′-TCCTCTCTACAC TCTCTGCGTTTA-3′. β-actin primer sequences were as follows: forward, 5′-AGCGAGCATCCCCCAAAGTT-3′ and reverse, 5′-GGGCACGAAGGCT CATCATT-3′. All primers were synthesized by Sangon Biotech (Shanghai, China).

Thermocycler conditions included an initial holding period at 95 °C for 10 min, followed by a 2-step PCR program of 95 °C for 15 s and 60 °C for 31 s for 40 cycles. Data were collected and quantitatively analyzed using the ABI Prism 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA).

β-Actin was used as an endogenous control. Gene expression levels were calculated as the difference in cycle threshold values (ΔC_T) between the target gene and β-actin. $\Delta\Delta C_T$ was estimated as the difference between the ΔC_T values of the test sample and the control. Relative expression of target genes was calculated as $2^{-\Delta\Delta CT}$.

2.6. Western blotting analysis

RA-FLS were cultured in the presence of IL-6/sIL-6R for 1 h, incubated with 4-H-CTX and/or MTX for 72 h, and subsequently harvested. Proteins were extracted in lysis buffer (5 μl 100 mM PMSF, 10 μl phosphatase inhibitor, and 1 μl protease inhibitor). The total protein concentration in the supernatant was determined using the bicinchoninic acid (BCA) protein assay kit (Sangon Biotech). The proteins (20 μg) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with primary antibodies overnight at 4 °C. Antibodies against RANKL and OPG were purchased from Abcam (Cambridge, UK), and the β-actin