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MDM2 promotes rheumatoid arthritis via activation of MAPK and NF-κB

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ABSTRACT

Murine double minute-2 (MDM2) has pleiotropic roles in immune activation and regulation. However, the role of MDM2 in rheumatoid arthritis (RA) remains unknown. We undertook this study to investigate the role of MDM2 in rheumatoid arthritis (RA). Fibroblast-like synoviocytes (FLS) were isolated from 25 patients with active RA and 25 patients with osteoarthritis (OA). FLS were stimulated in the presence or absence of IL-1β in vitro. Mice with collagen-induced arthritis (CIA) were treated with Nutlin-3a (100 mg/kg) or vehicle twice daily for 2 weeks. MDM2 expression was determined by Western blot. MDM2 was down-regulated by specific gene silencing. The concentrations of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) were analyzed using enzyme-linked immunosorbent assay (ELISA). The pathways of mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) were investigated by Western blot. Arthritis scoring and histological analysis were conducted. MDM2 expression was significantly higher in RA-FLS than in OA-FLS. MDM2 protein expression was positively correlated with disease activity of RA. MDM2 promoted the production of TNF-α, IL-6, MMP1 and MMP13 through MAPK and NF-κB pathways in RA-FLS. Nutlin-3a treatment decreased the arthritis severity and joint damage in CIA. Nutlin-3a also inhibited the activation of MAPK and NF-κB in arthritic joints. In conclusion, MDM2 inhibition exhibits anti-inflammatory activity and MDM2 might be a new therapeutic target for RA.

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1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by joint inflammation and cartilage erosions [1]. Fibroblast-like synoviocytes (FLS) contribute greatly to RA progression and joint destruction by initiating and regulating a number of pathways [2]. Molecular insights into FLS biology and related signaling pathways are needed to identify therapeutic targets [3].

Murine double minute-2 (MDM2), an E3 ubiquitin ligase, encodes a negative regulator of the p53 tumor suppressor [4]. MDM2 is an intracellular molecule with diverse biological functions and amplification of MDM2 occurs in multiple malignancies [5]. Furthermore, MDM2 promotes tissue inflammation and MDM2 inhibition has potent anti-inflammatory effects [6].

Nutlin-3a is a small-molecule antagonist that inhibits MDM2–p53 interactions and stabilizes the p53 protein, thereby inducing cell cycle arrest and apoptosis [7]. Inhibition of MDM2 attenuated neointimal hyperplasia via suppression of vascular proliferation and inflammation [8]. MDM2 links inflammation and tubular cell healing during acute kidney injury in mice [9]. Here, we undertook this study to investigate the role of MDM2 in rheumatoid arthritis (RA). In this study, we found that inhibition of MDM2 attenuated collagen-induced arthritis (CIA), suggesting a role for MDM2 in the pathogenesis of RA.

2. Materials and methods

2.1. Patients

Twenty-five patients who fulfilled the 2010 ACR/European League against Rheumatism (EULAR) classification criteria for RA [10] were enrolled from the Department of Rheumatology, Xiangya School of Medicine, Central South University. All patients had active disease with a 28-joint disease activity score (DAS28) more than 2.6. The patients with osteoarthritis (OA, $n = 25$) were included from the same Department as controls. This study was performed in accordance with the Declaration of Helsinki, and the protocol was approved by Ethics Committee of our Hospital. Written informed consent was obtained from all patients.

2.2. Synovial tissue collection and FLS culture

Closed needle was used to obtain the synovium samples from RA and OA patients [11]. All specimens were fixed in 10% neutral formalin and embedded in paraffin. Sections (5 μm) were cut serially and mounted on adhesive glass slides.

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FLS were isolated from the synovial tissues as previously described [12]. Fresh synovial tissues were digested in type I collagenase (Sigma-Aldrich, USA). The cells were cultured with DMEM-Ham's F-12 (Life Technologies, Shanghai, China), containing 20% fetal calf serum (Life Technologies, Australia) in a humidified 5% CO_2 incubator. FLS from passages three to six were used in this study.

2.3. MDM2 inhibition in RA-FLS

RA-FLS were pretreated with IL-1β (10 ng/ml, Minneapolis, USA) for 12 h. For MDM2 knockdown, RA-FLS were transfected with two different specific human MDM2 siRNAs (#1 and #2, Invitrogen, Life Technologies) using Lipofectamine 2000 reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Scrambled control siRNAs were used as control.

MDM2 siRNAs were added to RA-FLS in the presence of polybrene. In short, siRNAs and lipofectamine were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) separately and incubated for 10 min at room temperature. The diluted solutions were then mixed and incubated for 20 min at room temperature. Subsequently, the mixtures were added to each well. The culture plates were then incubated for 6 h at 37 °C in a CO_2 incubator. Then, the medium containing FBS was added. Cells or culture supernatants were subjected to the Western blot analysis or ELISA.

In addition, we tested the effects of MDM2 inhibitor Nutlin-3a (0, 5, 10, 20, 100 μM) on the cytokine production using the system as described above.

2.4. CIA induction and assessment

Male DBA/1 mice (8 weeks old) were purchased from the Shanghai Laboratory Animal Center and maintained under specific pathogen-free conditions. All experiments were approved by the Animal Use Committee of Xiangya School of Medicine, Central South University.

CIA was induced according to the previous report [13]. Chicken type II collagen in an emulsion with complete Freund's adjuvant (CFA; Hooke Laboratories) was initially immunized, and at 3 weeks chicken type II collagen in an emulsion with incomplete Freund's adjuvant was used for boosted immunization. Mice were observed and scored from the boost. The scoring for the severity of arthritis was conducted on a scale of 0–4 for each paw as follows [13], 0 = no clinical disease; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3 = entire paw inflamed and swollen; and 4 = severely inflamed and swollen paw or ankylosed paw.

2.5. Administration of Nutlin-3a

The mice with CIA were orally treated with Nutlin-3a (100 mg/kg, n = 10 mice) twice daily [14], starting from day 22 (1 day after boosted immunization) for 2 weeks. The control animals (n = 10 mice) were treated with vehicle (1% Klucel, 0.1% Tween 80) alone.

2.6. Western blot

The protein concentration was determined using the Bradford assay (BioRad). SDS sample buffer (5×) was added to the collected protein samples and boiled for 5 min. The proteins were then loaded onto a 10% SDS-PAGE gel and separated by electrophoresis. After transfer onto a nitrocellulose membrane, the membrane was blocked with 5% skim milk. The following primary antibodies were used: anti-p-ERK1/2, p-p38, p-JNK, NF-κB p65 and anti-GAPDH or Lamin B (all from Santa Cruz). The membrane was incubated with the primary antibodies overnight at 4 °C, followed by washing with PBST and incubation with peroxidase-labeled secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz Biotech). Protein visualization was achieved using enhanced chemiluminescence (ECL) and exposed to hyperfilm–ECL film (GE Healthcare Bio-Sciences, Australia).

2.7. Measurements of cytokines

Sera were obtained from anesthetized animals by retroorbital puncture at the end of the study. The levels of TNF-α, IL-6, MMP1 and MMP13 were measured by ELISA kits (R&D Systems) according to the manufacturer's instructions.

2.8. Histological assessment

Mice were sacrificed and the hind paws were fixed in 10% neutral formalin, decalcified by immersing in 10% EDTA solution for 20 days and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). The inflammation was evaluated as described previously [15]. Cartilage damage was examined using safranin-O staining and scored as follows [15]: 0, no destruction; 1, minimal erosion; 2, slight to moderate erosion in a limited area; 3, more extensive erosion; 4, general destruction.

2.9. Statistics

Data are expressed as mean ± SEM. All data were processed using SPSS 16.0. Statistical comparisons were performed using one-way analysis of variance or the Mann–Whitney U test. The significance of differences between 2 groups was determined using Student's unpaired t-test. $P < 0.05$ was considered significant.

3. Results

3.1. MDM2 was up-regulated in RA-FLS

The protein expression of MDM2 in RA-FLS was detected from 25 RA patients and 25 OA patients. Western blot analysis showed MDM2 was expressed in RA-FLS (Fig. 1A). The protein expression of MDM2 in RA-FLS was significantly enhanced compared with OA-FLS (Fig. 1B).

3.2. MDM2 protein expression was positively correlated with disease activity

Furthermore, we analyzed the correlations between MDM2 expression and clinical parameters including CRP, ESR, DAS28, RF titers, anti-CCP, 28TJC, 28SJC, and pain VAS. We found that MDM2 protein expression in RA-FLS was positively correlated with DAS28 ($r = 0.815$, $P = 0.005$). There were no significant correlations between MDM2 expression and other clinical features (all $P > 0.05$).

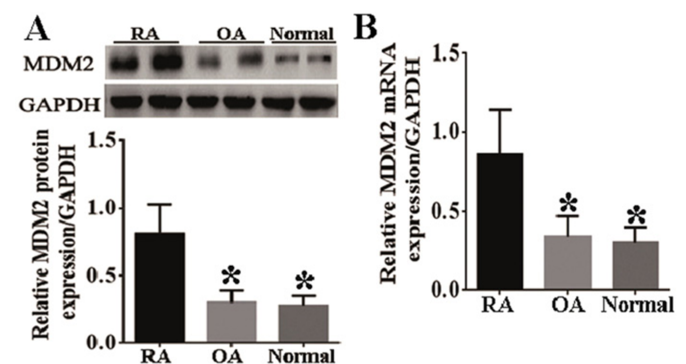

Fig. 1. The expression of MDM2 protein in fibroblast-like synoviocytes (FLS). A. MDM2 protein expression was detected by Western blot in FLS samples from RA, OA patients or healthy volunteers. B. The relative level of MDM2 mRNA expression was corrected by GAPDH. Data represent fold change of relative MDM2 expression normalized to GAPDH levels. *$P < 0.01$ vs RA.

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