Original Article
Effect of proteasome inhibitor on NF-κB and MMP-9 expression in synovial tissues of osteoarthritis rats

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Abstract: Nuclear factor (NF)-κB might participate in the pathogenesis of osteoarthritis (OA). Ubiquitin-proteasome system can activate NF-κB signal pathway, while over-expression of matrix metalloproteinase (MMP)-9 facilitates cartilage degeneration. This study thus generated one rat OA model, on which the effect of proteasome inhibitor MG-132 on synovial expression of NF-κB and MMP-9 was observed. A total of 80 male SD rats were randomly assigned into sham, model, solvent control and MG-132 groups. OA model was generated by resection of anterior cruciate ligament and meniscus medialis. 100 μl MG-132 solution (7 μg/ml) was weekly injected into kness joint. Rats were sacrificed 8 weeks after surgery and extracted for knee joint cartilage and synovial tissues, on which HE staining was performed. ELISA was also employed to describe TNF-α and IL-1β levels, while RT-PCR was used for measuring mRNA level of NF-κB p65, I-κB, MMP-9, and COX-2. OA model rats had elevated Mankin score and severe injury of cartilage and synovial tissues, with increase of TNF-α and IL-1β levels in joint cavity fluids and NF-κB, p65, I-κB, MMP-9, and COX-2 mRNA in synovial tissues (P<0.05). Compared to model group, MG-132 treatment alleviated cartilage and synovial injury, and significantly decreased TNF-α and IL-1β levels in joint cavity fluids, and NF-κB p65, I-κB, MMP-9, and COX-2 mRNA in synovial tissues (P<0.05). MG-132 could alleviate joint cartilage degeneration and synovial inflammation in OA rats, probably via down-regulating MMP-9 and inflammatory factor expression, and the blockade of IL-β/IKKβ/NF-κB signal pathway.

Keywords: Proteasome inhibitor, osteoarthritis, nuclear factor-kappa B, matrix metalloproteinase 9

Introduction

Osteoarthritis (OA) is one joint degenerative disorder, and is correlated with various factors including trauma, obesity, age, articular deformation, friction or congenital disorder. OA is clinically manifested as chronic joint pain, stiffness, swelling, deformation and limited activity [1, 2]. OA is one common articular disease in clinics. The pathological process mainly manifests as degradation of joint cartilage matrix degradation, in which the key factor is the metabolic alternation of chondrocytes. Both endogenous and exogenous pathways contribute to cartilage destruction, including degradation of extracellular matrix (ECM) by self-destruction, and ECM injury caused by infiltrative inflammatory cells, vascular and synovial tissues via articular fluids. Major factors for cartilage destruction are enzymatic degradation of ECM [3, 4]. Previous studies showed the important role of matrix metalloproteinase (MMPs) in articular cartilage injury [5, 6]. The major function of MMP-9 is keeping the dynamic balance between ECM degradation and synthesis. Elevated MMP-9 expression enhances ECM degradation of chondrocytes, thus facilitating degeneration. Nuclear factor (NF)-κB signal pathway may be involved in OA pathogenesis, and is activated by ubiquitin-proteasome system [7, 8]. Certain inflammatory factors can up-regulate COX-2 and MMP-13 expression via NF-κB signal pathway, thus affecting ECM synthesis and degradation. The inhibition of NF-κB signal pathway can alleviate inflammatory injury. NF-κB is activated by IKK kinase, and is translocated into the nucleus where it binds with DNA enhancer to regulate gene expression of multiple inflammatory factors [9, 10]. In eukaryotic cells, 26S proteasome complex can degrade multiple proteins involved in cellular signal transduction and apoptosis. In cell differ-
entiation and apoptosis, ubiquitin-proteasome system has an important role as proteasome inhibitor could suppress this pathway [11, 12]. Previous studies demonstrated that proteasome inhibitor could retard the progression of rat OA via inhibiting NF-κB p65 activated status and proteasome activity [13, 14]. This study established a rat knee joint OA model, on which the effect of proteasome inhibitor MG-132 on NF-κB and MMP-9 expressions in synovial tissues were observed, in order to investigate the related mechanism of proteasome inhibitor on the progression of rat OA.

Materials and methods

Animals

A total of 80 healthy male SD rats (6 month old, body weight 280~300 g) were provided by Laboratory Animal Center of Xi’an Jiaotong University (Certificate No., SYXK-2013-0025). Animals were singly housed in an SPF grade facility with food and water ad libitum. All rats had no trauma or swelling of knee joints, with normal extension and retraction functions. Animals were randomly assigned into sham, model, solvent control and MG-132 groups (N=20).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Hong-Hui Hospital, Xi’an Jiaotong University College of Medicine.

Drugs and reagents

Proteasome inhibitor MG132 was purchased from Sigma (US) and was diluted in DMSO before use. Hydrate chloral and paraformaldehyde were purchased from Kemiou Chemical (China). Rabbit anti-I-κB p65, anti-I-κB, anti-MMP-9 and anti-COX-2 antibody was purchased from Boster (China). Trizol kit and reverse transcription kit were purchased from Invitrogen (US). Horseradish peroxidase (HRP) labelled goat anti-rabbit secondary antibody was purchased from CST (US). Primers were provided by Aibosi (US). ELISA kits for TNF-α,

Table 1. Primer sequence

<table>
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<th>Target gene</th>
<th>Sequence (5’-3’)</th>
<th>Fragment length (bp)</th>
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<td>NF-κB p65</td>
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<td>286</td>
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<td></td>
<td>Reverse CAAGATCAATGGCAACACGG</td>
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<tr>
<td>I-κβ</td>
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<td></td>
<td>Reverse GCCTCACACCCTCTTCTA</td>
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<tr>
<td>MMP-9</td>
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<td>331</td>
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<tr>
<td></td>
<td>Reverse TTGTGTTGTCGCCAATTGA</td>
<td></td>
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<tr>
<td>COX-2</td>
<td>Forward AACGAGTACCGCAAACGC</td>
<td>370</td>
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<td></td>
<td>Reverse GCTGAGGATCTGGGACGT</td>
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<tr>
<td>GADPH</td>
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<td>492</td>
</tr>
<tr>
<td></td>
<td>Reverse ACAAGTGGTCAATGAGGC</td>
<td></td>
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</tbody>
</table>

Figure 1. Morphology of rat knee joint cartilage. A. Sham group; B. Model group; C. Solvent control group; D. MG-132 group.
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IL-1β were purchased from Jiancheng Bio (China). Bradford protein quantification kit was purchased from Huyu Bio (China). Fluorescent polypeptide substrate Z-LLVY-AMC, Z-LLE-AMC and 7-amino-4-methyl-coumarin was provided from Sigma (US).

Animal model

SD rats were firstly acclimated for one week, followed by treadmill exercise (r=4 cm, 4 rpm) for 1 week before surgery, and at every other day (30 min each) after surgery. Knee joint OA model was established by resection of anterior cruciate ligament and meniscus medialis as previously described methods [15]. Before surgery, rats were fasted for 8 h, followed by anesthesia by 10% hydrate chloral. Using the right side as the surgical side, skin was sterilized then. Sham rats were incised from articular cyst but without removal of anterior cruciate ligament and meniscus medialis. In the other three groups, after opening articular cyst, soft tissues were separated for the entry into cavity. Anterior cruciate ligament and meniscus medialis were removed by surgical blade. The articular cavity was sutured after rinsing. After surgery, penicillin was given intramuscularly without fixation of surgical site. General motor activity and wound healing were observed. In MG-132 group, 100 μl MG-132 solution (7 μg/ml) was injected into the knee joint cavity 24 h after surgery. Equal volume of DMSO (0.1% v/v) was applied in solvent control group. Drug delivery was performed weekly for 8 consecutive weeks.

ELISA for TNF-α and IL-1β levels in knee joint cavity fluid

Articular fluid was collected from knee joint, and was centrifuged at 14,000 g (r=10 cm, 3500 r/min) for 15 min. Supernatants were saved and tested for TNF-α and IL-1β levels following instruction of ELISA kits.

General observation of knee joint cartilage

8 weeks after surgery, rats were sacrificed to dissect knee joint. Any pathological injury including joint fluid aggregation, synovial swelling, surface/edge injury or articular cartilage was observed.

HE staining for morphology of synovial tissues

8 weeks after surgery, rats were sacrificed to dissect knee joint. Synovial tissues were separated and fixed in paraformaldehyde, followed by paraffin embedding and sectioning. Hematoxylin-eosin staining was performed, followed by coverslip mounting and observation under the light field microscope.

Mankin score

Cartilage tissues were stained by hematoxylin-O staining, followed by light field microscopy. Mankin score was evaluated semi-quantitatively for evaluating disease condition of OA as previously described [16]. The category includes staining intensity of O staining, articular cartilage structure, tidal line and chondrocytes, in a scale from 0 to 13 (most severe injury).
RT-PCR for mRNA level of NF-κB p65, I-kB, MMP-9 and COX-2

Total RNA was extracted from synovial tissues. cDNA was synthesized from RNA by reverse transcription. RT-PCR was performed using Trizol. UV spectrometry was employed for quantification. Primer sequences were listed in Table 1. Amplified products were analyzed by agarose gel electrophoresis in triplicates and were expressed as relative expression level, which was shown by the ratio of target gene gray value against GADPH. Analysis was performed by Bio-Rad gel imaging system and Quantity One 4.31 software.

Fluorescent spectrometry for 20S proteasome activity

Rat knee joint was dissected to separate synovial tissues, which was homogenized and centrifuged at 14 000 r/min for 10 min. Supernatant was collected for 60 min centrifugation to obtain crude extract of 20S proteasome subunit from the precipitation. Total protein concentration was measured by Bradford kit following manual instruction. Using fluorescent polypeptide substrate Z-LLE-AMC and Z-LLVY-AMC, release of 7-amino-4-methyl-coumarin (AMC) from proteasome was measured using 380 nm excitation and 460 emission wavelengths. The activity of proteasome was presented as AMC release content.

Statistical methods

SPSS20.0 software was used for data analysis. Measurement data was firstly tested for normal distribution. Those fitted normal distribution were presented as mean ± standard deviation (SD). The comparison among multiple groups was performed by one-way analysis of variance (ANOVA). Paired comparison within groups was carried out by LSD test. A statistical significance was defined when P<0.05.

Figure 3. Red-O staining and Mankin score of rats and analysis of Mankin score. A. Sham group; B. Model group; C. Solvent control group; D. MG-132 group; *, P<0.05 compared to control group; #, P<0.05 compared to model group; Δ, P<0.05 compared to solvent control group.
Results

General condition and observation of rat knee cartilage

All rats had satisfactory wound healing after surgery, without infection in knee joint, or stiffness or half dislocation. In a general observation, sham group had peral-white joint cartilage with smooth and complete edge. Model and solvent control group, however, had swelling joint cartilage with rough surface and friction, plus severe injury on the loading surface of femoral medial condyle. Drug treatment group had smooth surface of cartilage without swelling. Surface smoothness and brightness were all better than model or solvent control group (Figure 1).

Morphology of synovial tissues

HE staining revealed no edema in synovial tissue mesenchyme of sham group, which had regular arrangement of cells without inflammatory infiltration and minor hyperplasia of small vessels. Model group and solvent control group had significantly fewer cells in synovial tissues, with thickening of lining layer, fibrosis, infiltration of inflammatory cells and hyperplasia of intra-synovial small vessels. Drug treatment thickened lining layer cells, decreased inflammatory cell infiltration, with minor hyperplasia of synovial micro-vessels and fibrosis (Figure 2).

Mankin score

O staining showed evenly distributed ECM of sham group, whilst model group and solvent control group had unevenly distributed ECM. Drug treatment group had darker staining compared to model solvent control group, indicating fruitful cartilage matrix (Figure 3).

TNF-α and IL-1β levels in joint cavity fluid

Comparing to control group, model rats had significantly elevated TNF-α and IL-1β levels in knee joint cavity fluids (P<0.05). Drug treatment group had significantly depressed TNF-α and IL-1β levels compared to model or solvent control group (Figure 4).

RT-PCR for mRNA level of NF-κB p65, I-κβ, MMP-9 and COX-2

Compared to control group, model or solvent control group had significantly elevated mRNA levels of NF-κB p65, I-κβ, MMP-9 and COX-2 (P<0.05). Drug treatment group had remarkably decreased mRNA levels of all those genes (P<0.05 compared to solvent control group, Figure 5).

Activity of 20S proteasome

Compared to control rats, model or solvent control rats had significantly elevated activity of proteasome activity (P<0.05). Compared to solvent control group, drug treated rats had sup-

Figure 4. Expression level of TNF-α (left) and IL-1β (right) in knee joint cavity fluid. *, P<0.05 compared to control group; #, P<0.05 compared to model group; Δ, P<0.05 compared to solvent control group. A. Sham group; B. Model group; C. Solvent control group; D. MG-132 group.
pressed proteasome activity (P<0.05, Figure 5).

Discussion

OA is one common articular disease featured with degradation of articular cartilage ECM. Critical factor of disease progression is the alternation of chondrocyte metabolism. Pathological basis of OA is cartilage degeneration and ECM degradation. With further studies on MMPs and cytokines, it has been found that multiple MMPs and cytokines were involved in articular cartilage destruction in a complex regulatory networks rather than single factor [17]. MMP-9 is one proteinase degrading cartilage ECM. Under normal conditions, MMP-9 secretion is maintained at homeostasis. The amount of secretion is of critical importance for maintaining integrity of cartilage. MMP-9 level was elevated to different extents in OA patient’s serum [18]. MMP-3 may facilitate collagen, destruction of ECM, swelling of joint, lower resistance and eventually cartilage destruction via regulating the activity of other enzymes such as MMP-1 or MMP-9 [19]. Recent study found various NF-kB related genes, including inflammatory factors such as TNF-α, IL-6, IL-8 and ICAM-1, plus other genes for prostaglandin, NO production, anti-apoptosis and proliferation. NF-kB can cause ECM degradation via inducing MMP and cytokine production, further leading to articular cartilage injury. In synovial tissues of OA patients, NF-kB p65 is abundantly distributed. It has important roles in arthritis animal model. NF-kB signal pathway is activated before clinical manifestation of symptoms. In early phase of arthritis, NF-kB was observed in nucleus of chondrocytes, while activated NF-kB p65 was found in lining cells and inflammatory synovial cells. The injection of IKKβ on normal rats leads to joint tissue swelling [20, 21]. In fibroblast-like synovial cells, activated NF-kB could facilitate cell proliferation, and the release of MMPs and cytokines. Moreover, the production of MMP and inflammatory factor IL-1β require activation of NF-kB. Both TNF-α and IL-1β enhance I-κβ func-

![Figure 5](image-url). Relative mRNA level of NF-κB p65, I-κβ, MMP-9 and COX-2. *, P<0.05 compared to control group; #, P<0.05 compared to model group; Δ, P<0.05 compared to solvent control group. A. Sham group; B. Model group; C. Solvent control group; D. MG-132 group.

![Figure 6](image-url). Proteasome activity of 20S subunit in synovial tissues. *, P<0.05 compared to control group; #, P<0.05 compared to model group; Δ, P<0.05 compared to solvent control group. A. Sham group; B. Model group; C. Solvent control group; D. MG-132 group.
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...leading to NF-κB transcription and I-κBα degradation, further accelerating expression of MMP, TNF-α and IL-1β [20, 22].

Proteasome inhibitor functions on proteasome. Ubiquitin-proteasome system is the major pathway activating NF-κB. Within proteasome complex, NF-κB p105, which was one precursor protein, was degraded into p50 in an ubiquitin-dependent manner. Phosphorylated I-κB was degraded by ubiquitin-proteasome system. The activation of NF-κB facilitates chemokines and adhesion molecules expression in chondrocytes and synovial tissues. Proteasome inhibition can suppress NF-κB activity, prevent I-κBα degradation, thus alleviating inflammatory injury [12]. Ubiquitin-proteasome system also elevates protein expression of tumor suppressor p53, p16 and p27, and induces caspase family activation and synthesis of death receptor [23]. MG-132 could prevent the degradation of ubiquitin-linked target protein by 26 s proteasome subunit, and inhibit the degradation of abnormal proteins by ubiquitin-proteasome system [13]. Both in vitro and in vivo confirmed it oncogenic effect as it could induce apoptosis of osteosarcoma cell line MG63 [24]. This study revealed that proteasome inhibitor MG-132 significantly alleviate injury of articular cartilage and synovial tissues, suppressed TNF-α and IL-1β levels in joint cavity fluids, and decreased mRNA expression of NF-κB p65, I-κB, MMP-9 and COX-2, indicating that MG-132 could improve cellular metabolism of articular chondrocytes and synovial tissues, and tis down-regulation on NF-κB activity, prevention of I-κBα degradation, decrease of inflammatory factor TNF-α and IL-1β secretion, and down-regulation of MMP-9, all of which are important for cartilage ECM degradation and retard such process. The down-regulation of COX-2 mRNA inhibited injury of inflammatory mediators such as prostaglandin on chondrocytes. This study has not performed detailed mechanism analysis of MG-132 on NF-κB signal pathway, probably due to the inhibition on IL-1β/IKKβ/NF-κB/MMP-9/COX signal pathways. This study compared the activity of 20S proteasome body in all groups, and showed the correlation between peptidyl glutamyl hydrolase and chymotrypsin activity in synovial tissues and cellular metabolism. MG-132 inhibits the activity of 20S proteasome in synovial tissues, suggesting that injection of MG-132 inside the articular cavity can be absorbed by synovial tissues, where it can alleviate inflammatory injury by inhibiting 20S proteasome unit activity and decreasing NF-κB activity.

Conclusion

MG-132 effectively alleviate cartilage degeneration of OA rats, as well as synovial inflammation, probably via down-regulating MMP-9 or inflammatory cytokine expression, and the blockade of IL-1β/IKKβ/NF-κB signal pathway.

Disclosure of conflict of interest

None.

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