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Effect of IL-1β on apoptosis of synovial cells in rheumatoid arthritis rats *via* the NF-κB pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of interleukin-1β (IL-1β) in the apoptosis of synovial cells in rheumatoid arthritis (RA) rats, and to explore the underlying mechanism.

MATERIALS AND METHODS: The apoptosis of the synovial cells in RA rats in the IL-1β group and the control group was analyzed by scoring under an electron microscope. The expressions of cleaved-poly (ADP-ribose) polymerase (PARP), PARP and anti-apoptosis gene products in synovial cells of IL-1β treated RA rats were explored as well. Meanwhile, the expressions of B-cell lymphoma 2 (Bcl-2), Bcl-xL, and Active-Caspase3 in the synovial cells of RA rats with IL-1β treatment were evaluated by the Western blotting. To further clarify the relationship between IL-1β and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway in the synovial cells of RA rats, the expressions of NF-κB regulated the gene products of matrix metalloproteinase-3 (MMP-3), MMP-9, cyclooxygenase-2 (Cox-2), and vascular endothelial growth factor (VEGF) in synovial cells of RA rats after that we investigated the treatment with IL-1β (was investigated). In addition, the expression of NF-κB in the synovial cells of RA rats treated with IL-1β was determined.

RESULTS: The results showed that, compared with the control group, IL-1β treatment significantly increased the number of apoptotic cells. This meant that IL-1β treatment could promote the apoptosis of the synovial cells ($p<0.05$). IL-1β treatment significantly promoted the expression level of cleaved-PARP ($p<0.05$). However, it remarkably reduced the expressions of Bcl-2 and Bcl-xL ($p<0.05$). Meanwhile, the level of the active-Caspase3 in the synovial cells of RA rats treated with IL-1β was significantly enhanced ($p<0.01$). In comparison with the control group, the IL-1β group exhibited significantly elevated expressions of NF-κB-regulated gene products in the synovial cells of RA rats ($p<0.01$). Besides, the positive markers of the activated NF-κB were detected in the synovial cells of RA rats in the IL-1β group and the control group. The results demonstrated that they were mainly located in the nucleus of the IL-1β group.

CONCLUSIONS: IL-1β can promote the apoptosis of the synovial cells in RA rats via the NF-κB pathway.

Key Words:
IL-1β, NF-κB pathway, RA rats, Synovial cells, Apoptosis.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune and inflammatory disease characterized by synovitis-induced arthritis[1]. RA synovial fibroblasts are able to regulate osteomalacia and bone erosion by secreting the pro-inflammatory cytokines[2]. The synovial cells closely interact with the inflammatory factors in RA. The mechanism of action is probably as follows. After the synovial cells secrete the pro-inflammatory cytokines[3], the inflammatory factors stimulate the synovial cells to produce the matrix-degrading enzymes, including matrix metalloproteinase (MMP) and cyclooxygenase-2 (Cox-2)[4]. The subsequent release of prostaglandins leads to the destruction and degeneration of the extracellular matrix in cartilage. Previous studies[5,6] have reported that interleukin-1β (IL-1β) can induce massive apoptosis and

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extensive mitochondrial dysfunction of synovial cells. During this process, the synthesis of the reactive oxygen species and apoptosis occurs[7].

The intracellular signaling is a complex signal communication network. It regulates all basic biological functions of the cells[8]. Current studies have found that the signaling pathway goes wrong in the synovial cells in RA[9]. The effective treatment strategies for arthritis can target various cell signaling pathways, thereby effectively alleviating the inflammation in synovial cells, as well as systemic reactions.

Previous studies have indicated that the pro-inflammatory factors involved in the onset and progression of RA are regulated by the transcription factor nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB)[10]. In addition, the transcription factors in the cell signaling pathway of B-cell lymphoma 2 (Bcl-2)/Bax family proto-oncogenes, including NF-κB, TNF-α, and IL-1β, are able to stimulate cell apoptosis[11]. After that, this pathway activates Caspase-3, eventually inducing apoptosis[12]. Therefore, the aim of this study was to verify whether IL-1β affected the apoptosis of the synovial cells in rats with RA through the NF-κB pathway.

Materials and Methods

Main Experimental Reagents

2, 6, 10, 14-tetramethylpentadecane was purchased from Beyotime (Shanghai, China); Freund's complete adjuvant from Sigma-Aldrich (St. Louis, MO, USA); Cleaved-poly (ADP-ribose) polymerase (PARP), PARP, Bcl-2, Bcl-xL, Active-Caspase3, MMP-3, MMP-9, Cox-2, vascular endothelial growth factor (VEGF), and β-actin antibodies from Abcam (Cambridge, MA, USA).

Experimental Animals

The Sprague-Dawley (SD) male rats weighing 250-300 g were purchased from the Hubei Experimental Animal Research Center. They were kept under the conditions with the temperature of 18-24°C and humidity of 50%. All rats were given free access to food and water. This study was approved by the Animal Ethics Committee of People's Hospital of Ningxia Hui Autonomous Region Animal Center.

Establishment of RA Model in Rats

The RA rat model was established as previously mentioned above[13]. The experimental rats were kept in cages at 4-8°C for 20 d, with humidity of 80-90% and light-dark cycles for 12 h. On the next day, they were treated with pentobarbital sodium for anesthesia. Subsequently, 10 mg/mL Freund's complete adjuvant was subcutaneously injected between the second and third toes of the right foot. During 3 days of observation, the right ankle of the experimental rats showed an acute inflammatory swelling within 24 h. In addition, extensive arthritis occurred in forelimbs and contralateral limbs within 24 h. The main clinical symptoms were inflamed lymph nodes and arthritis, indicating the successful establishment of the RA model in rats.

Isolation and Culture of Synovial Cells

The articular cartilage of the right forelimb ankle bone of the model rats was first collected, and the primary synovial cells were isolated. The cartilage tissues were digested with 1% proteinase at 37°C for 2 h and 0.2% collagenase at 37°C for 4 h. A total of 2×10^5 cells were seeded into cell culture plates and cultured for 24 h in an incubator with 5% CO_2 at 37°C.

Evaluation of Apoptosis Via Electron Microscopy

The serum-starved synovial cells were first treated with 10 ng/mL IL-1β, and the control group was set up. Subsequently, the samples were prepared into ultra-thin sections and evaluated under an electron microscope. The number of apoptotic cells with the morphological characteristics of death was determined by scoring 100 cells from 20 fields of view.

Western Blotting

The synovial cells were lysed, and the albumin from bovine serum (BSA) was chosen as a standard to quantify the protein concentration. The extracted protein samples were separated by dodecyl sulfate and sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with 1% gelatin-Phosphate-Buffered Saline with Tween 20 (PBST) for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were washed with PBST for three times and incubated with horseradish peroxidase-coupled secondary antibody for 2 h. Then, the membranes were washed again with PBST three times. The immuno-reactive bands were developed by diaminobenzidine (DAB; Solarbio, Beijing, China), and the density of each band was measured by light densitometry.

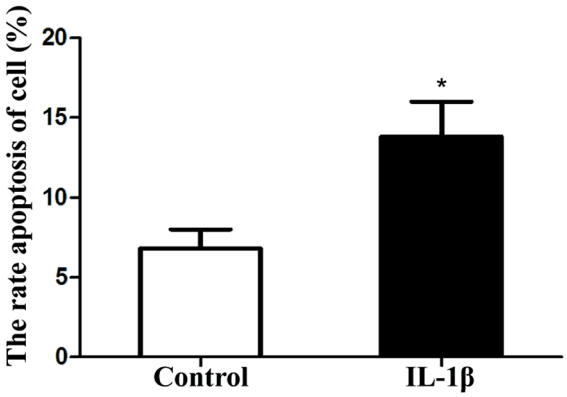

Figure 1. Impact of IL-1β treatment on apoptosis of the synovial cells in RA rats. *p<0.05, indicating a significant difference.

Determination of NF-κB Activation

The effect of IL-1β treatment on NF-κB nuclear translocation was detected by immunocytochemistry. Briefly, the synovial cells were first seeded in glass plates and treated with 10 ng/mL IL-1β. After incubation, the cells were fixed with cold methanol for 10 min and pre-incubated with serum for 10 min. After that, the cells were incubated with primary antibody (p65 antibody) at 4°C overnight. After washing with PBS twice, the cells were treated with the dual system APAAP complex at room temperature for 30 min. Next, they were thoroughly washed with TBS and re-stained with new fuchsine at room temperature for 30 min. The cells were then washed, air dried, and placed in glycerol gelatin. Finally, the cells were observed with an Axiophot 100 optical microscope.

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for all statistical analyses. The One-Way analysis of variance (ANOVA), followed by the post-hoc test (Least Significant Difference) was used to compare the difference between the two groups. The experimental data were expressed as mean ± standard deviation. p<0.05 was considered statistically significant.

Results

Impact of IL-1β Treatment on Apoptosis of Synovial Cells in RA Rats

To explore the role of IL-1β in the apoptosis of the synovial cells in RA rats, the apoptosis of the synovial cells in the IL-1β group and the control group was analyzed by scoring under the electron microscope, respectively. The results revealed that, compared with the control group, the IL-1β group showed a significantly increased number of apoptotic cells. This implied that IL-1β treatment promoted the apoptosis of the synovial cells (p<0.05) (Figure 1).

Influence of IL-1β Treatment on PARP Expression in Synovial Cells of RA Rats

It is universally known that the enhanced caspase-mediated DNA repair enzyme Cleaved-PARP is an important marker of cell degeneration and apoptosis. In this study, we focused on the effect of IL-1β treatment on the protein expressions of Cleaved-PARP and PARP in the synovial cells of RA rats. As shown in Figure 2, IL-1β treatment

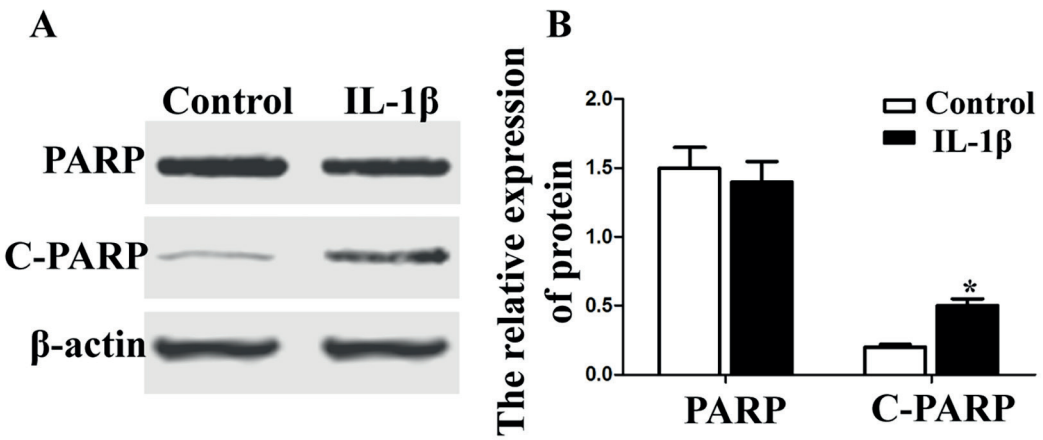

Figure 2. Influence of IL-1β treatment on PARP expression in the synovial cells of RA rats. **A,** Western blotting results showed that IL-1β treatment significantly increased C-PARP. **B,** Semi-quantitative analysis of the protein levels of PARP and C-PARP (*p<0.05, indicating a significant difference).

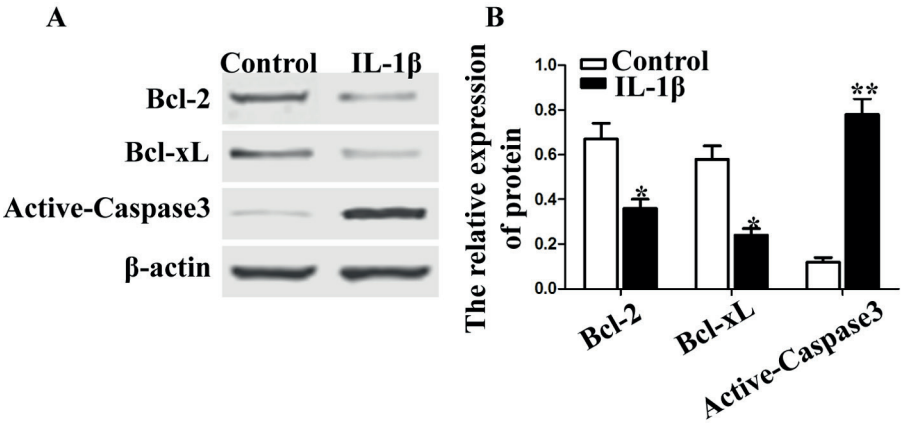

Figure 3. Effect of IL-1β treatment on the expression of apoptotic inhibitor in the synovial cells of RA rats. **A,** IL-1β significantly decreased Bcl-2 and Bcl-xL, but increased Active-Caspase 3. **B,** Semi-quantitative analysis of the protein levels of Bcl-2, Bcl-xL and Active-Caspase 3 (*p<0.05, indicating a significant difference. **p<0.01, indicating an extremely significant difference).

significantly up-regulated the level of Cleaved-PARP when compared with the control group (p<0.05). However, no evident changes were observed in the total amount of PARP (p>0.05).

Effect of IL-1β Treatment on the Expression of Apoptotic Inhibitor in Synovial Cells of RA Rats

To determine whether IL-1β regulated the expression of anti-apoptotic gene products in the synovial cells of RA rats, the expression of apoptotic inhibitor in the synovial cells of RA rats treated with IL-1β was detected via Western blotting. As shown in Figure 3, compared with the control group, IL-1β significantly reduced the protein expressions of Bcl-2 and Bcl-xL (p<0.05). However, the level of Active-Caspase3 in the synovial cells of RA rats treated with IL-1β was significantly enhanced (p<0.01).

Impact of IL-1β Treatment on NF-κB Regulated Gene Products in Synovial Cells of RA Rats

To further elucidate the relationship between IL-1β and NF-κB pathway in the synovial cells of RA rats, the expression of the gene products regulated by NF-κB after IL-1β treatment in synovial cells of RA rats was explored. As shown in Figure 4, IL-1β

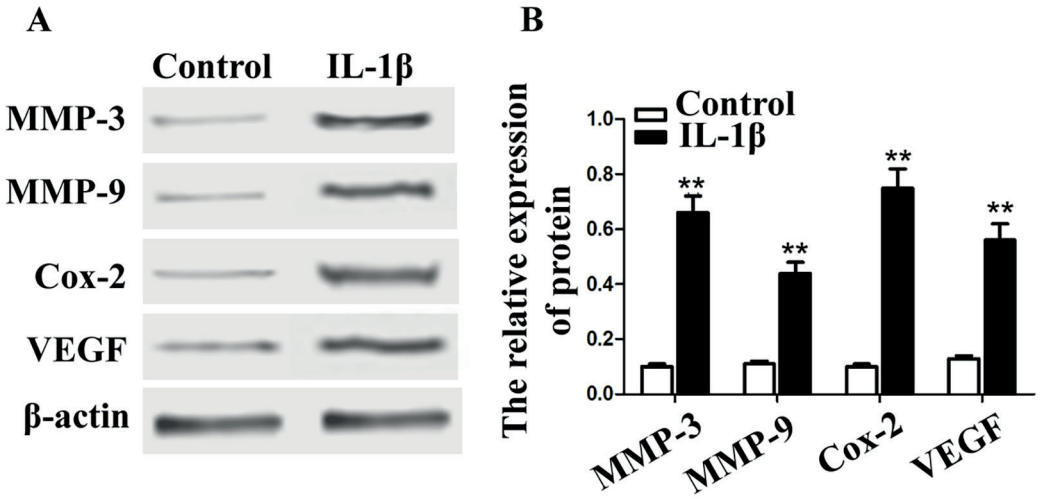

Figure 4. Impact of IL-1β treatment on the expressions of MMP-3, MMP-9, Cox-2, and VEGF in synovial cells of RA rats. **A,** IL-1β remarkably enhanced expressions of MMP-3, MMP-9, Cox-2 and VEGF. **B,** Semi-quantitative analysis of the protein levels of MMP-3, MMP-9, Cox-2 and VEGF (**p<0.01, indicating a significant difference).

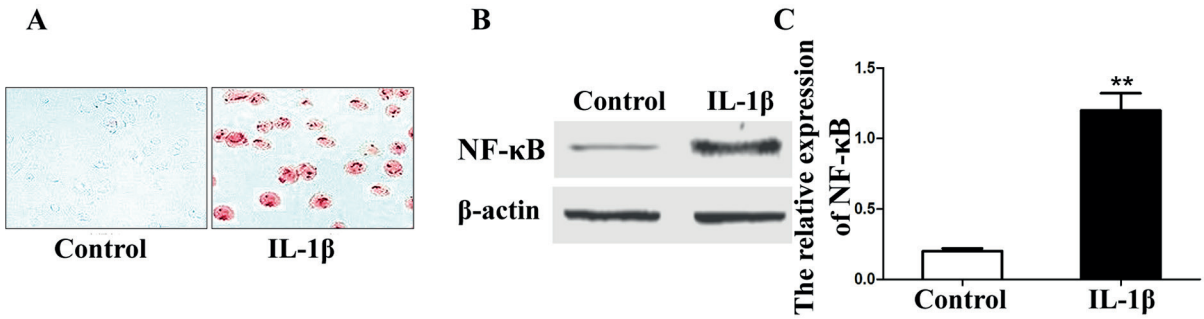

Figure 5. Influence of IL-1β treatment on NF-κB expression in synovial cells of RA rats. **A,** Effect of IL-1β on NF-κB nuclear translocation detected by immunocytochemistry (´ 200). **B,** Effect of IL-1β on NF-κB protein expression determined *via* Western blotting, **C,** NF-κB was increased after treatment with IL-1β (**p<0.01, indicating a significant difference).

treatment group showed remarkably enhanced expressions of MMP-3, MMP-9, Cox-2, and VEGF in the synovial cells of RA rats when compared with the control group (p<0.01).

Influence of IL-1β Treatment on NF-κB Expression in Synovial Cells of RA Rats

Multiple studies have found that NF-κB plays an important role in regulating the expression of the inflammatory cytokine genes. The mechanism is possible as follows. After NF-κB phosphorylation, the fragment is transferred into the nucleus, thereby binding to and activating the target genes[20]. In this study, the expression of NF-κB in the synovial cells of RA rats treated with IL-1β was explored. As shown in Figure 5, the synovial cells of RA rats treated with IL-1β generated positive markers of the activated NF-κB when compared with the control group. Meanwhile, these markers were mainly located in the nucleus.

Discussion

RA is a common inflammatory arthropathy, which can lead to joint impairment and disability after improper treatment[14,15]. RA is characterized by synovial hyperplasia and inflammation, chondropathy, and bone erosion, as well as systemic inflammatory complications[16]. The aim of this study was to determine whether the effect of IL-1β on the apoptosis of the synovial cells with RA was regulated by NF-κB-mediated signal transduction pathway and NF-κB-regulated gene expression.

In this study, we found that IL-1β could induce the apoptosis of the synovial cells with RA. Meanwhile, IL-1β significantly promoted the expression of Cleaved-PARP, reduced the expressions of Bcl-2 and Bcl-xL, and promoted the expression of the Active-Caspase3. The pro-inflammatory and pro-apoptotic effects of the synovial cells with RA after IL-1β stimulation were related to the up-regulation of NF-κB specific gene products. The activation and transfer of NF-κB from the cytoplasm to the nucleus could be clearly observed in the synovial cells of RA rats treated with IL-1β. Furthermore, IL-1β treatment could significantly promote NF-κB expression in the synovial cells with RA.

Researches have pointed out that NF-κB is activated by receptor activator of NF-κB ligand (RANKL) and IL-1β. Its expression, as well as binding to receptor RANK, are necessary for osteoclast formation[17]. Although they are not essential for (developmental) the development of the osteoclastogenesis, they can enhance the inflammation-induced bone loss[18]. Currently, Li et al[19] have shown that the small peptides that prevent IKKα and IKKβ catalytic subunits from binding to IKKγ regulatory subunit are capable of inhibiting the inflammation-induced bone loss. These findings further clarify the role of NF-κB in osteoclast formation. However, it remains unclear which of the two IKK catalytic subunits play a more critical role in basal osteoclast formation and inflammation-induced bone loss. Pro-inflammatory cytokine IL-1β can induce osteoclast formation of IkkαAAMB progenitor cells together with RANKL. Therefore, although IKKα contributes to RANKL-induced differentiation *in vitro*, its function *in vivo* is not essential for activating the other factors of the NF-κB pathway driven by IKKβ. These factors may be derived from osteoblasts during normal bone development. Meanwhile, these factors may include TNF-α and IL-1β during inflammation.

Authors[20,21] have illustrated that the pro-inflammatory cytokine IL-1β can mediate the cartilage degeneration and apoptosis of the synovial cells in RA in both humans and animals. Cytokine-mediated apoptosis of the synovial cells has been confirmed to be vital for the pathogenesis of RA. IL-1β activates ubiquitous transcription factor NF-κB and up-regulates the pro-inflammatory cytokines of Cox-2 and MMPs, thereby degrading ECM macromolecules. This may eventually result in cartilage degeneration and arthritis[22]. The activation of NF-κB potentially associates the inflammation with hyperplasia in RA[23]. It has been uncovered that NF-κB inhibitor reduces the severity of the joint swelling in arthritic mice[24]. As a result, NF-κB may serve as an important target in the preventive treatment of RA. In this study, our results revealed that IL-1β stimulated several NF-κB-regulated genes, including the anti-apoptotic gene products (Bcl-2 and Bcl-xL), pro-apoptotic protein Active-caspase3, matrix degradation genes (MMP3 and MMP9), as well as angiogenesis and inflammation gene products (VEGF and Cox-2). Our findings[25] indicated that the expression of Bcl-2 and Bcl-xL were regulated by NF-κB.

Conclusions

This study uncovers the effect of IL-1β on the apoptosis of the synovial cells in RA rats. Our results indicated that IL-1β promoted the apoptosis of the synovial cells in RA rats via the NF-κB pathway.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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