Effects of Triptolide on the Expression and Activity of NF-κB in Synovium of Collagen-induced Arthritis Rats*

TU Shenghao (涂胜豪), HU Yonghong (胡永红), ZENG Keqin (曾克勤), ZHANG Mingmin (张明敏), LAI Xianyang (赖先阳), ZHANG Weichen (张玮琛)
Department of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary: The expression and activity of NF-κB in the synovium of collagen-induced arthritis (CIA) rats was detected in order to investigate the possible therapeutic effects of triptolide on rheumatoid arthritis (RA). The experimental Wistar rat model of CIA was set up by intradermal injection of emulsion of bovine collagen Ⅱ and the successful rate of setting-up models was evaluated by arthritis index (AI). Rats were grouped randomly into three groups: normal, model and treatment group. The expression of TNF-α and IL-6 in synovial fluid was detected by ELISA, and the expression and activity of NF-κB in synovium by immunohistochemistry method and by electrophoretic mobility shift assay (EMSA) respectively. As compared with normal group, the expression of TNF-α and IL-6 in synovia ($P<0.05$), and the expression and activity of NF-κB ($P<0.05$) in synovium were increased in model group. There was statistical difference in above-mentioned indexes between model group and treatment group. Triptolide may play a protective role in RA via downregulating the expression and activity of NF-κB in synovium.
Key words: triptolide; collagen-induced arthritis; NF-κB; synovium; cytokine

Rheumatoid arthritis (RA) is characterized by synovial proliferation and influx of a variety of inflammatory cells. The process of disease progression results in bone and joint destruction. Accumulation of many proinflammatory cytokines and chemokines in the synovium of RA patients is involved in the inflammatory and destructive process. These proteins include cytokines such as TNF-α, IL-6 and IL-8; adhesion molecules such as E-selectin, intercellular adhesion molecule 1; and enzymes such as the nitric oxide synthase and cyclooxygenase. The expression of all these proteins depends on transcriptional activator nuclear factor κB (NF-κB). Therefore, targeting the activation of NF-κB-dependent transcription by pharmacologic agents may prove to be quite useful in the treatment of RA and other inflammatory disorders. We report here triptolide, an active compound from *Tripterygium wilfordii Hook F* (TWHF), downregulates the expression and activity of NF-κB in synovium of collagen-induced arthritis (CIA) rats.

1 MAERIALS AND METHODS

1.1 Animals and Treatment

Fifty male rats, weighing from 130－150 g, were purchased from Experimental Animal Center of Tongji Medical College. Ten rats were randomly selected as the normal group. Forty rats were immunized with Bovine collagen Ⅱ (BCⅡ) in Freund's complete adjuvant (FCA) as earlier described[1]. Briefly, BCⅡ was completely dissolved in 0.1 mol/L glacial acetic acid at final concentration of 4 mg/mL and then stored at 4 ℃ overnight. BCⅡ solution was mixed and emulsified in an equal volume of FCA. All these procedures were made aseptically. Rats were immunized by intradermal injection at the 5 locations of the back and neck. Each point was injected with 0.05 mL BCⅡ emulsion. Booster injections were administered 15 days later. The rats in normal group were injected with physiological saline in the same way as indicated above. On the day 15 after the second immunization with CII, severity of arthritis was evaluated. The clinical severity of arthritis was quantified according to a scoring system[1]. The rats with 6 score-arthritis were selected and randomly divided into treatment group and model group ($n=10$ in each group). Triptolide was purchased from Fujian Institute of Medical Science (China). Triptolide powder was dissolved in 5 % propylene glycol (100 μg triptolide per mL), filtrated, and then stored at 4 ℃. The rats were treated with triptolide (0.04 mL/100 g, intramuscularly, every third day) at hind leg commencing on Day 30. The rats in model group and normal group received respectively 0.5 % propylene glycol or physiological saline by the same way described above.

1.2 Synovium Separation

The rats were killed at 31st day after the triptolide injection. The synovium was obtained as previously described[2,3]. For the immunohistochemistry, the pieces of synovium in right knee joints of triptolide group were cut, washed two times with phosphate buffered saline and fixed in 10 % methanal. For the detection of NF-κB, the synovium in left knee joints was obtained. After

TU Shenghao, male, born in 1965, Associate Professor
* This project was supported by a grant from National Natural Sciences Foundation of China (No. 30070961).

removing synovium, the joints were washed with 1 mL physiologic saline, and then the physiologic saline was collected for detection of TNF-α and IL-6.

1.3 Enzyme-linked Immunosordent Assay (ELISA)

TNF-α and IL-6 in the synovial fluid were measured using commercially available ELISA kits (BioSource International Company, China) according to the test protocol.

1.4 Immunohistochemistry

Synovium slide was deparaffinized through xylene and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 3 % H_2O_2-methanol for 15 min at 37 ℃. The slide was washed with distilled water and immersed in PBS for 5 min. Antigen retrieval was routinely performed in a microwave for 30 min. The slide was washed 3×3 min and then incubated with goat serum at 37 ℃. The serum was removed 15 min later. The antigen was bond with a prime antibody in a humidified chamber at 4 ℃ overnight which was monoclonal rabbit anti-rat NF-κB/p65 antibody at 1 : 200 dilution. Slides prepared by substituting PBS for the primary antibody served as the negative control. After a brief wash in PBS, the slide was treated with goat anti-rabbit antibody for 45 min at 37 ℃. The slide was washed 3×3 min and then incubated with the third antibody labeled by streptomycin-horseradish peroxidase for 45 min at 37 ℃. The slide was developed in diaminobenzedine solution (DBA) and then counterstained with hematoxylin. The values of grayscales were quantified with automatic image analyzer.

1.5 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of the synovium were prepared as previously described[4] and quantified using Bradford's method. EMSA was performed using a commercial kit (Promega, USA). The NF-κB oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Santa Cruz, USA) was end-labeled with [γ-^{32}P] ATP (Bejing Free Biological Medical Company, China). The non-denatuing 50 g/L polyacrylamide gel was ran in 0.25×TBE buffer at a current of 18 mA for 40—60 min. The gel was vacuum-dried and exposed to X-ray film for 24 h at −70 ℃. Autoradiograms were quantified using imagequant analysis software.

1.6 Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Statistical significance was determined by one-way ANOVA using SPSS11.5.

2 RESULTS

2.1 Production of TNF-α and IL-6

The levels of TNF-α and IL-6 in synovial fluid of CIA mice were significantly increased as compared with normal group ($P<0.01$). Triptolide could suppress the production of TNF-α and IL-6 (table 1).

2.2 Expression of NF-κB p65 in Synovium

The expression levels of NF-κB p65 in the synovium in CIA mice were higher than in normal mice. The cells positive for NF-κB p65 were lymphocytes, macrophages and fibroblasts which were distributed in synovial lining layer and synovial subintima. The staining existed in both cytoplasm and nucleus. The staining in nucleus was agglomerate and deep. After treatment with triptolide, the level of NF-κB staining was decreased markedly (table 2 and fig. 1).

Table 1 Production of TNF-α and IL-6 (pg/mL, $\bar{x}\pm s$)

Groups	TNF-α	IL-6
Normal	19.42±4.23	19.38±2.24*
Model	44.17±8.94*	88.69±10.56*
Treatment	22.54±4.76	48.67±5.97

* $P<0.01$ as compared with treatment group

2.3 Activity of NF-κB in Synovium

The activity of NF-κB in model group was higher than in normal group ($P<0.05$). The activity of NF-κB in treatment group was almost as

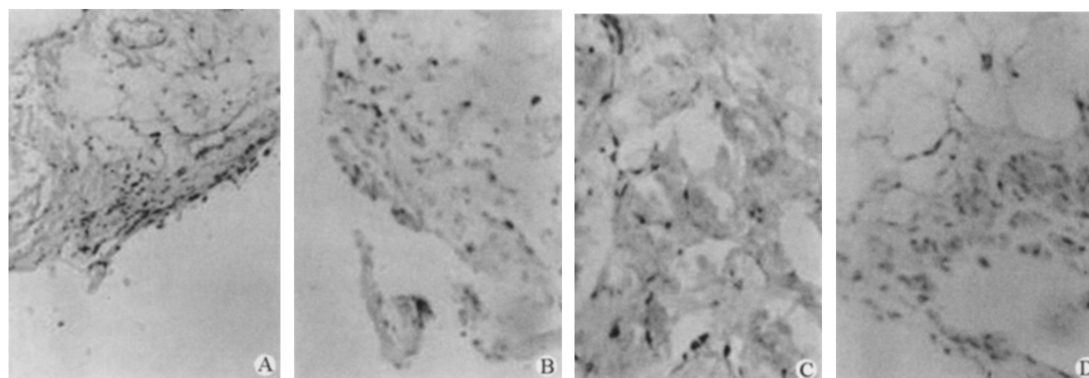

Fig. 1 Expression of NF-κB (p65)
A: Negative control slide; B: Positive staining of NF-κB was not found on sections of normal control group; C: In model group, NF-κB was extensively expressed in both nuclei of cytoplasm of cells; D: After treatment with triptolide, positive staining of NF-κB was detectable occasionally on the sections of normal control group

high as that in normal group ($P>0.05$, table 2 and fig. 2).

Table 2 Expression and activity of NF-κB in synovium ($\bar{x}\pm s$)

Groups	Expression of NF-κB(p65)	Activity of NF-κB
Normal	90.27±9.38	13.85±3.56
Model	169.15±10.72*	25.63±4.67*
Treatment	87.06±7.61	16.47±4.21

* $P<0.05$ *vs* treatment group

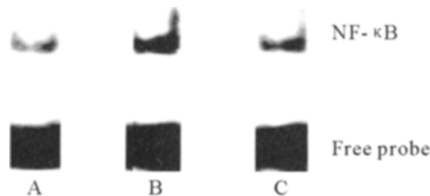

Fig. 2 Activity of NF-κB in synovium
Lane A: Normal group; Lane B: Model group; Lane C: Treatment group

3 DISCUSSION

Because CIA shares a number of pathological, genetic, and immunologic features in common with RA, the murine CIA model is widely used to study disease mechanisms and potential therapies for RA. The Chinese herbal remedy TWHF has been known for thousands of years as a therapeutic agent against RA and other autoimmune diseases[5]. Triptolide has been identified as a major active compound in TWHF. Previous studies have shown that triptolide can exert both immunosuppressive and anti-inflammatory activities. The actions were partially attributed to the inhibition of cyclooxygenase (COX)-2, TNF-alpha, IL-1 beta, NO and prostaglandin E_2 in rheumatoid fibroblasts and other cell types[6,7]. In our previous study, it was shown that triptolide could influence the proliferation of synovium in CIA rats by induction of apoptosis[8]. However, we still understand little mechanisms by which triptolide is used to treat rheumatism diseases.

NF-κB is a collective name for dimeric transcription factors comprised of the proteins that include RelA (p65), c-Rel, RelB, NF-κB1 (p50) and NF-κB2 (p52). The most abundant form found in stimulated cells is the RelA/NF-κB1 (p65/p50) heterodimer, often referred to as a "classic" NF-κB. In unstimulated cells, NF-κB resides in the cytoplasm in a latent form, and must translocate to the nucleus to function. The cytoplasmic retention of NF-κB is provided by its interaction with inhibitory proteins known as IκB. Stimulation leads to a phosphorylation-targeted proteasomal degradation of IκB, allowing the active NF-κB to enter the nucleus and initiate transcription[9].

Activated NF-κB has been detected in human synovial tissue on the early stage of joint inflammation, as well as in specimens obtained at the late stage of the disease. It was also found in different animal models of RA, such as CIA in mice, adjuvant arthritis in rats and pristine-induced arthritis in rats[9], NF-κB is a pivotal regulator of inflammation in RA. It plays an important role both at the stage of initiation and the stage of perpetuation of chronic inflammation in the disease. NF-κB controls the expression of cytokines TNF-α and IL-6 that are key mediators in the pathogenesis of RA. We demonstrated that tirptolide suppressed the NF-κB p65 expression and NF-κB activity in synovial tissue. Our studies also revealed that the levels of TNF-α and IL-6 in synovial fluid, as well as in serum (data not published), were reduced after the treatment of triptolide. The suppression of NF-κB activation may be responsible for the decrease of production of TNF-α and IL-6 in synovial fluid. These results may partially accounts for the triptolide therapeutic effects on RA.

REFERENCES

1 Larsson P, Kleinau S, Holmdahl R *et al*. Homologous type Ⅱ collagen-induced arthritis in rats. Characterization of the disease and demonstration of clinically distinct forms of arthritis in two strains of rats after immunization with the same collagen preparation. Arthritis Rheum, 1990,33(5):693

2 Dayer J M, Krane S M, Russell R G G *et al*. Production of collagenase and prostaglandine by isolated adherent rheumatoid synovial cells. Proc Natl Acad Sci USA, 1976,73(3):945

3 Goto M, Saasno M, Yamanaka H *et al*. Spontaneous production of an interleukin 1-like factor by cloned rheumatoid synovial cells in long-term culture. J Clin Invest, 1987,80(3):786

4 Edgar S, Partrick M, Mitheal M *et al*. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res, 1989,17: 6419

5 Lipsky P E, Tao X L. A potential new treatment for rheumatoid arthritis: thunder god vine. Semin Arthritis Rheum, 1997,26:713

6 Zhuo H F, Niu D B, Xue B *et al*. Triptolide inhibits TNF-alpha, IL-1 beta and NO production in primary microglial cultures. Neuroreport, 2003,14(7):1091

7 Tao X L, Schulze-Koops H, Ma L *et al*. Effects of Tripterygium wilfordii hook F extracts on induction of cyclooxygenase 2 activity and prostaglandin E2 production. Arthritis Rheum, 1998,41:130

8 Hu Y H, Weng G M, Lu F E *et al*. Experimental investigation on triptolide apoptosis of synoviocytes in collagen induced arthritis rats. Chin J Rheumatol (Chin), 2004, 8: 131

9 Makarov S S. NF-kappaB in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction. Arthritis Res, 2001,3:200

(Received June 21, 2004)