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Original Article

Mechanism of Herbal Acupuncture of *Clematis Mandshurica Maxim.* Water Extract by Stimulation of Sinsu (BL 23) Loci Subcutaneously as Dual Inhibitor of Proinflammatory Cytokines on Adjuvant Arthritis in Rats

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Objective : Based on immunological mechanisms, this study examined whether subcutaneous (s.c.) injection of *Clematis mandshurica* Maxim. water extract (CMA) has anti-inflammatory effects, and its effect on TNF-α, IL-1 and IL-10 release from synoviocytes on adjuvant arthritis (AA) in the rat.

Methods : Complete Freund's adjuvant was used to induce AA in rats. Synoviocytes were separated by the method of collagenase and DNase digestion. Synoviocytes proliferation was assayed by 3-(4, 5 dimethylthiazol 2 yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. TNF-α, IL-1 and interleukin-10 (IL-10) production of synoviocytes was measured with ELISA. The expression of IL-10 mRNA of synoviocytes was determined using RT PCR.

Results : There were significant secondary inflammatory reactions in AA rats, accompanied by the decrease of body and immune organs weight simultaneously. Synoviocytes proliferation of AA rats significantly increased, and the levels of TNF-α and IL-1 in supernatants of synoviocytes in AA rats were also elevated compared with the sham group. The administration of CMA (2, 5, 10 mg/kg, s.c.) reduced the above changes significantly. In contrast to TNF-α and IL-1, IL-10 production and the level of its mRNA of synoviocytes in AA rats apparently decreased. CMA (2, 5, 10 mg/kg, s.c.) markedly increased IL-10 in synoviocytes at protein and transcription level.

Conclusion : The results indicate that CMA has a beneficial effect on rat AA due to modulating inflammatory cytokine production of synoviocytes, which play a crucial role in the pathogenesis of this disease.

Key Words : *Clematis Mandshurica* Maxim. water extract, adjuvant arthritis, *Sinsu* (BL 23)-loci, synoviocytes, TNF-α, IL-1, IL-10

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint swelling, synovial membrane inflammation, and cartilage destruction. Recent evidence suggested that proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), increased in the synovial tissue and synovial fluid in RA, and played a pivotal role in the pathology of RA. Evidence has been provided that anti-TNF-α antibodies and soluble TNF-α receptors were effective in RA[1]. Previous investigation suggested that IL-1 contributed to synoviocyte self-proliferation and subsequent cartilage destruction in adjuvant arthritis (AA)[2]. On the other hand, the anti-inflammatory activities of interleukin-10 (IL-10), which suppresses TNF-α production, are well known. It was reported that systemic IL-10 treatment suppressed the development of collagen induced

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arthritis in rats[3]. Thus, down-regulation of proinflammatory cytokines such as TNF-α and IL-1, and up-regulation of interleukin-10 might be a new and rational approach for therapy of RA. Although there are a few anti-rheumatic drugs such as antibodies, soluble receptors, receptor antagonists and recombinant IL-10 showing effectiveness on RA, these proteins were limited due to host immune response, rebound of symptoms, a short half-life, and cost[4,5].

Herbal acupuncture of *Clematis mandshurica* Maxim. water extract (CMA) has been used as a potent anti-inflammatory agent for arthritis and inflammation for centuries in Asian countries, including Korea and China[6]. For scientific study, Choi et al. (2002) and Jung et al. (2001) have recently reported that natural herbal products extracted from three herbs including *Clematis mandshurica* on articular cartilage is protective for osteoarthritis (OA) in *in vivo* models and OA-like degeneration of the articular cartilage and synovial tissue[7,8].

It was discovered as a potent inhibitor of TNF-α and IL-1 production in lipopolysaccharide (LPS)-stimulated human monocytes and phytohemagglutinin M-stimulated human lymphocytes[9-12]. Based on the immunological dysfunction in RA and the immunological feature of CMA, we examined whether subcutaneous (s.c.) administration of CMA has anti- inflammatory effects in rat adjuvant arthritis (AA) in the present study. Furthermore, in order to clarify the immunological activity of CMA, we also investigated its effect on TNF-α , IL-1 and IL-10 release from synoviocytes in AA rats.

Materials and Method

1. Materials

1) Animal and reagents

Male Sprague-Dawley (SD) rats weighing 180-220 g were obtained from the Biochemical Animal Department, Dongguk Oriental Medical Experimental Animal Center. All animals were maintained at a controlled temperature (22±2°C), and a regular light/dark cycle (7:00 to 19:00 h, light), and all animals had free access to food and water. CMA was kindly provided by the Department of Acupuncture. Lipopolysaccharide (LPS), collagenase type II, trypsin, and 3 (4, 5 dimethylthiazol 2 yl) 2, 5-diphenyltetrazolium bromide (MTT), ELISA kits of TNF-α , IL-1 and IL-10 were all purchased from Sigma (St. Louis, MO). RPMI 1640 medium was purchased from Gibco (CA, USA). The primers of IL-10 and GAPDH were synthesized by Bioneer Co. (Daejon, Korea).

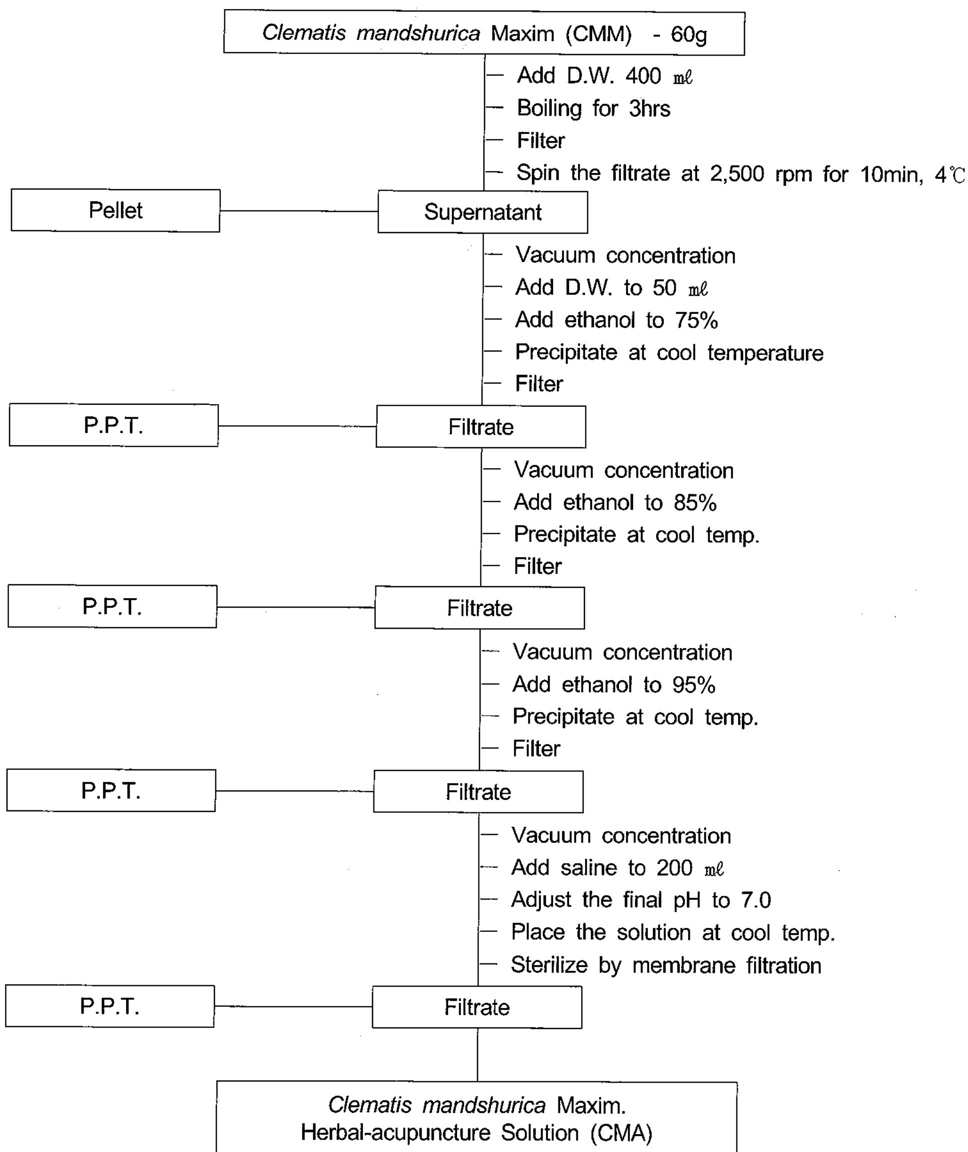
2) Preparation of Clematis mandshurica Maxim. (CMM) herbal acupuncture water extract

60g CMM was put in a flask with 400 mℓ distilled water (DW), boiled for 3-hrs & filtrated. After centrifugation (4℃, 2,500 rpm), pellets were discarded and supernatants vacuum concentrated by rotary evaporator (BUCHI RE121, Switzerland). Supernatant was supplemented with DW to 50 mℓ and filtrated by membrane filter (0.22 μm, Whatman, Germany). Pellets were treated with ethanol to 99.9%, precipitated at cool temperature and filtrated. Finally, pellets were treated with saline to 200 mℓ, final pH adjusted to 7.0, the solution placed at cool temperature and sterilized by membrane filtration. CRA for application in cell culture were suspended in 0.5% methylcellulose at concentrations of 2, 5 or 10 mg/kg/0.2 mℓ.

2. Methods

1) AA induction and clinical evaluation

Rat AA was induced as previously described [13-16]. Briefly, rats were immunized on day 0 by intradermal injection of Freund's complete adjuvant (FCA), containing 10 mg heat-inactive



Scheme 1. Preparation of *Clematis mandshurica* Maxim. Herbal-Acupuncture Solution (CMA)

BCG in 1 ㎖ paraffin oil, into the left hind paw in 0.1 ml for each rat. As a sham control, the same volume of paraffin oil alone was given into the left hind paw. Right hind paw volume was determined with MK-550 volume meter (Muromachi Kikai, Japan) before immunization (basic value, day 0) and repeated on days 14, 17 and 21. Right hind paw swelling was expressed as increase in hind paw volume in milliliters calculated by subtracting the basal value from the hind paw volume measured at all times considered. The polyarthritis severity was graded on a scale of 0-4[13]: grade 0, no swelling; grade 1, isolated phalanx joint involvement; grade 2, involvement

of phalanx joint and digits; grade 3, involvement of the entire region down to the ankle; and grade 4, involvement of entire paw, including ankle. The maximum joint score was 12, excluding the evaluation of the left hind paw for each rat.

2) CMA herbal-acupuncture treatment

CMA in 0.5% methylcellulose at concentrations of 2, 5 or 10 mg/kg/0.2 ㎖ was administered by both Sinsu (BL 23) loci of 0.2 ml aliquots once daily from the day when the adjuvant was given to day 21 of the experiment. As a positive control, leflunomide (20 mg/kg) was intragastrically (i.g.) administrated[17].

3) Isolation and culture of synoviocytes

Synoviocytes were prepared by collagenase and DNase digestion of small minced membranes as described[18,19], with minor modifications. In brief, synovial tissues were digested in RPMI 1640 medium containing 5% fetal bovine serum (FBS), 1 ㎎/㎖ collagenase A (Sigma, USA), and 0.15 mg/ml DNase I (Sigma, USA) for 2 h at 37°C, 5% . After incubation, the tissue was pipetted through sterile nylon mesh into a sterile centrifuge tube. Cells were then washed three times with RPMI 1640 plus 5% FBS. Cells were cultured in 24 well plates at 5 × 105 cells/well in RPMI 1640 containing 5 ㎎/ℓ LPS, 100 IU/㎖ penicillin, and 100 ㎍/㎖ streptomycin, at 37°C, 5%. Supernatants were harvested at 48 h and stored at 70°C until measurement for the production of TNF-α , IL-1 and IL-10.

4) Synoviocytes proliferation assay

Synoviocytes were isolated according to the above method[20]. Then, the cells were suspended in RPMI-1640 medium with 15% FBS at a concentration of 2 × 109 cells/ℓ . The cell suspension of 100 ㎕ were added to 96-well flat-bottomed culture plates and incubated at 37 °C, 5% for 24 h. After the cells adhered, the cultures were replaced by RPMI-1640 medium without bovine serum and incubated at 37°C, 5% CO2 for 24 h. Aspiring RPMI-1640 medium without bovine serum and adding 100 ㎕ RPMI-1640 medium with 15% FBS, the cultures were incubated at 37°C, 5% CO2 for 66 h. A 10 ㎕ sample of MTT (5 g/ℓ) was added to each well, oscillated for 1 min on oscillator at 37°C and 5% CO2 for 2 h continuously. After incubation, the cultures were centrifuged (760 × g, 10 min). The supernatants were aspired, 120 ㎕ of isopropanol (containing HCl 0.04 M) was added to each well and oscillated for 30 sec again. The absorbance (A) was measured at 570 nm. The results were described as the average of A.

5) Measurement of proinflammatory cytokines in culture supernatants of synoviocytes

The levels of TNF-α, IL-1, and IL-10 in cell culture supernatants were measured using commercial ELISA kit[12]. Measurements were performed in duplicate.

6) RT-PCR assay for IL-10 mRNA in synoviocytes

Synoviocytes in different groups were isolated and cultured according to the above method. Cells were harvested at 48 h postculture and kept at 70°C until RNA extraction. Total cellular RNA was extracted using RNA easy kits (Invitrogen, USA). To test the efficacy of reverse transcriptase, RT-PCR was performed for GAPDH mRNA[21]. Briefly, the first strain of cDNA was synthesized by reverse transcriptase and pooled. The resulting cDNA samples were adjusted to PCR buffer conditions and were run for PCR simultaneously. The following primers were used[20,22]: IL-10 sense 5'-AGA GCC CCA

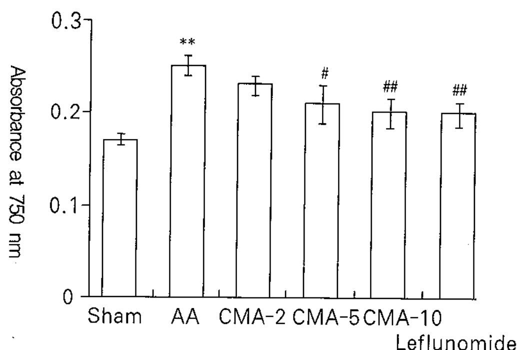


Fig. 1. Effects of CMA on synoviocytes proliferation of AA rats.
CMA (5, 10 mg/kg, s.c.) significantly decreased the response of synoviocytes proliferation.
n=9, mean±S.D.; **P<0.01 compared with Sham; #P<0.05, ##P<0.01 compared with AA.

GAT CCG ATT TT-3' and antisense 5'-CAT CAA GGC GCA TGT GAA CT-3' and GAPDH sense 5,-CGA TGC TGG GCG TGA GTA C-3' and antisense 5'-CGT TCA GCT CAG GGA TGA CC-3'. Cycling conditions were as follows: 30 sec of denaturation at 94°C, 2 min of annealing at 57°C and 30 sec of elongation at 72°C for 35 cycles. About 10 $\mu\ell$ of reaction mixture were loaded to 1% agarose gel containing 0.5 ug/ml ethidium bromide for electrophoresis; and the gel was then placed under ultraviolet ray for semiquantitative detection.

7) Statistical analysis

Unless stated otherwise, data are expressed as mean ± S.D., evaluated using an ANOVA followed by Dunnett's test. P<0.05 was considered statistically significant.

Results

1. Effects of CMA on secondary arthritis in AA rats

In our preliminary study, inflammatory polyarthritis was induced in all immunized rats. The peak incidence occurred on day 14 after immunization. Treatment with CMA (2, 5 and 10

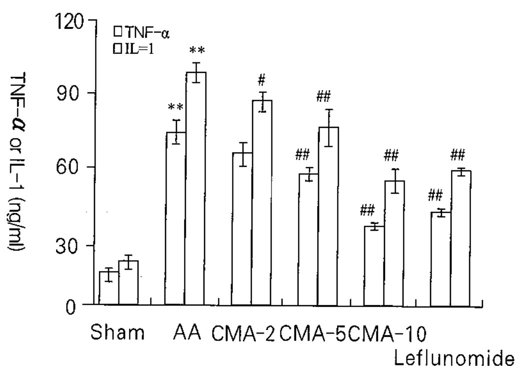


Fig. 2. Effects of CMA on TNF-α and IL-1 levels in the synoviocytes supernatants of AA rats.
CMA (2, 5, and 10 mg/kg, s.c.) evidently decreased the production of TNF-α and IL-1 by synoviocytes.
n=9, mean±S.D.; **P<0.01 compared with Sham; #P<0.05, ##P<0.01 compared with AA.

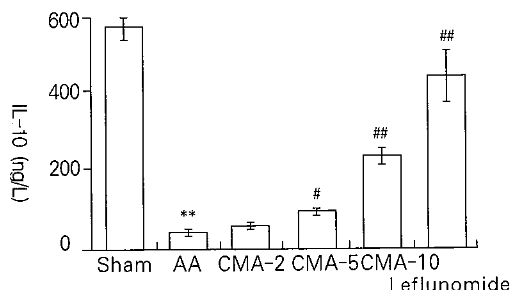


Fig. 3. Effects of CMA on IL 10 levels in the synoviocytes supernatants of AA rats.
CMA (5, 10 mg/kg, s.c.) significantly increased IL 10 secretion by synoviocytes. n=9, mean±S.D.; **P<0.01 compared with Sham; #P<0.05, ##P<0.01 compared with AA.

mg/kg, s.c.) and leflunomide (20 mg/kg, i.g.) attenuated the right hind paw swelling and polyarthritic symptoms from day 14 to day 21 after immunization (data not shown). The increase of body weight was comparable to immunized and nonimmunized rats.

2. Effects of CMA on synoviocytes proliferation of AA rats

The synoviocytes proliferation of AA rats was determined at day 21 after immunization. It was found that there were increases of synoviocytes proliferation in AA rats. The administration of CMA (5, 10 mg/kg, s.c.) and leflunomide (20 mg/kg, i.g.) obviously decreased the response of synoviocytes proliferation (Fig. 1).

3. Effects of CMA on proinflammatory cytokines produced by synoviocytes

Synoviocytes from AA rats released a higher level of TNF-α and IL-1 than that from the sham rats. However, the level of IL-10 in culture supernatants of synoviocytes from AA rats apparently decreased compared with the sham. In the present study, we demonstrated that CMA (2, 5 and 10 mg/kg, s.c.) decreased the production of TNF-α and IL-1, while it increased IL-10 secretion by synoviocytes (Fig. 2 and Fig. 3).

4. Effect of CMA on IL 10 mRNA expression in synoviocytes

Representative levels of IL-10 mRNA in synoviocytes were shown in Fig. 4. The expression

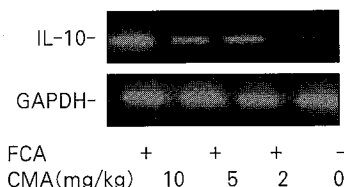


Fig. 4. Effect of CMA on IL 10 mRNA expression in synoviocytes of AA rats.
CMA apparently increased IL-10 mRNA expression in synoviocytes of AA rats. n=3. Representative RT PCR data is presented.

of IL-10 mRNA in synoviocytes of AA rats was lower than that of synoviocytes in sham rats. Treatment with CMA apparently increased IL-10 mRNA expression in synoviocytes.

Discussion

Although previous studies showed that CMA could prevent the onset and progression of AA and collagen-induced arthritis[7,8], the mechanisms of CMA on arthritis were not fully understood. Because AA is often used as an animal model of RA in the evaluation of antirheumatic drugs[23], the present study sought to elucidate the effects and mechanisms of CMA on AA. The results demonstrated that CMA markedly inhibited joint swelling and the index of polyarthritis, and significantly reduced the histological degrees of joint injury. An increase of body weight was also apparently improved in the rats treated with CMA. The above results suggested that CMA would be effective in rat AA.

Synoviocytes are the ultimate targeting cells of pathologic change of arthritis[24]. In particular, cytokines secreted from synoviocytes, such as TNF-α and IL-1, are considered major determinants in the perpetuation of arthritis[25]. TNF-α contributed to the induction of proinflammatory cytokines as well as matrix metalloproteinases that could be responsible for joint destruction, whereas IL-1 might be more important in processes leading to cartilage and bone destruction and in limiting mechanisms involved in cartilage repair[1,2]. Thus, various strategies to block their action are now being clinically applied. In our study, the proliferative response of synoviocytes of AA rats was significantly increased compared with the sham group. Meanwhile, synoviocytes of AA rats also released a higher level of TNF-α and IL-1 than those of the sham rats. Administration of CMA significantly reduced the proliferation of synoviocytes and inhibited the production of TNF-α and IL-1 by synoviocytes. Therefore, CMA appeared to ameliorate the secondary inflammatory reaction of AA by reducing proliferation of synoviocytes and inhibiting secretion of TNF-α and IL-1 from activated synoviocytes.

In contrast to the functions of TNF-α and IL-1, IL-10 inhibited the production of Th1 and proinflammatory cytokines, including TNF-α, IL-1, IFN-r, and IL-2[3,26]. The administration of IL-10 suppresses progression of arthritis in animal models[26]. In patients with active RA, treatment with anti-IL-10 results in an increase in the production of TNF-α and IL-1 by rheumatoid synovium in vitro[26]. Previously, Cho et al.[20] and Wei et al.[22] reported a similar result using cyclosporin, showing that IL-10 release is decreased from synoviocytes of AA rat was, and the same observation was also made in our experiment. Administration of CMA apparently increased IL-10 secretion by synoviocytes. We wondered why the secretion of IL-10 increased while the proliferation of synoviocytes was reduced after treatment with CMA. Therefore, the effect of CMA on IL-10 mRNA in synoviocytes was also investigated. The result shows that CMA significantly increased the expression of IL-10 mRNA in synoviocytes of AA rats, indicating that CMA protected inflammatory joints from destruction partly by means of promoting IL-10 production at transcription levels.

In the present study, administration of CMA significantly increased the IL-10 at protein and transcription levels in synoviocytes of adjuvant arthritis rats. As we know, the synoviocytes

consisted of macrophage-like synoviocytes and fibroblast-like synoviocytes, which interact in the development of arthritis[22].

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