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원저

Effect of *Ulmus davidiana Planch* (Ulmaceae) on T-lymphocyte-producing cytokines such as IL-2, IL-6, and IFN-γ production in collagen-induced arthritis of rats

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Abstract

Objective : The effect of Ulmus davidiana Planch(UD), which has long been known to have anti-inflammatory and protective effects on damaged tissue, inflammation and bone among other functions, on the development of type II collagen (CII)-induced arthritis (CIA) in rats was studied.

Methods : Male rats were immunized with an emulsion of 200 μg of CII and complete Freund's adjuvant (CFA). The rats were then given intraperitoneal stimulation of Ulmus davidiana Planch herbal acupuncture(UDHA)or saline during the experiment. When compared with rats treated with saline as control, UDHA at doses of more than 20μg/100 g rat once a day for 7 days inhibited the ability of inguinal lymph node cells to produce T cell cytokines interleukin-2, interleukin-6, IFN-γ when the cells were obtained from rats 14 days after immunization and cultured in vitro with CII.

Results : When rats were injected intraperitoneally, UD -treated group and control group rats did not differ significantly when low doses of UD was given to rats.

Conclusion : The recommended dose of UD in the management and treatment of rat CIA will be more than 20μg/100 g, which is two-firth of human therapeutic dose. From the results, it was concluded that the effect of UDHA is dependent of dosage.

Key words : Rheumatoid arthritis, Ulmus davidiana Planch (Ulmaceae), type II collagen (CII)-induced arthritis (CIA), IL-2, IL-6, IFN-γ

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I. Introduction

Immunization with type II collagen (CII) is well known to be able to induce inflammatory polyarthritis in rats and susceptible strains of mice[1-2]. Although immune mechanisms that include both humoral and cellular immunity to CII have been implicated in the pathogenesis of the disease[3-4], there is much evidence that anti-CII antibodies play an important role in the initiation of the diseases [5,6.. Since CII-induced arthritis (CIA) in rats and mice is well-known to have both clinical and histological similarities to human RA[1-2], these models has been widely used to evaluate anti-arthritic drugs[5-8]. It is seemed that RA is an autoimmune disease in the cartilage and synovial membrane. In the initiation and development of this disease, immunological and inflammatory pathways are critical, and the antigen specific T cell responses to CII are especially important. Many investigators have tested the hypothesis that the modulation of immune responses to CII, especially the T cell mediated response, can depress the incidence and the severity of arthritis. Treatments using cytokines and anti-cytokine antibodies have been shown up- and down-regulate the development of arthritis induced by CII and CFA in rodents[9-10].

In this paper, we have evaluated UD into the joint for its effectiveness on immune responses to CII in the rat CIA. In an attempt to gain further insight into the mode of action of UD, we also investigated the effects of UD on the incidence and development of arthritis in rat CIA with 4 different regimens: (1) started prior to a primary immunization, (2) started on the day of a primary immunization.

II. Materials and methods

1. Materials

1) Plant material

The stem barks of U. davidiana were collected from Mt. Phal-gong, Kyungbuk Province, South Korea in May 2002, and identified by Professor Kap-Sung Kim, College of Oriental Medicine, Dongguk University, South Korea. Fresh stems were dried in a dark, well ventilated place. The voucher specimen (No. UD-W-57) is deposited in the Herbarium of this college.

2) chemicals and biochemicals

Rats were allowed at least 1 week to adapt to the environment (25±3℃, 55±5% humidity and a 12 h light/dark cycle) and were used at 7 weeks of age. All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea). Radiochemicals were from Amersham International Co. (Seoul, Korea). Culture flasks and dishes were obtained from Nunc (Roskilde, Denmark). Unless otherwise stated, all other chemicals were purchased from Sigma (CA, USA). Media and sera for cell culture were purchased from Jeil Biotech Services (Taegu, Korea).

2. Induction of arthritis

CII collagen (Sigma, St. Louis, MO) extracted from bovine articular cartilage was dissolved overnight at 4℃ in 0.1 M acetic acid at 2.0 mg/ml, after which the solution was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI, USA) in an ice-cold water bath.

Arthritis was induced by an intradermal injection of 0.1 ml of the cold emulsion into the base of the tail. Rats were boosted subcutaneously with same volume of the emulsion 21 days later. As the CFA control, 0.1 M acetic acid emulsified in an equal volume of CFA alone was injected to control rats using the same schedule. The onset of arthritis was considered to be present when erythema and swelling were detected in at least one joint.

3. Preparation of LN cell suspension

Rats were killed under ether anesthesia on day 24 after immunization with CII. Inguinal lymph nodes were removed aseptically and pressed through a 60 gauge steel mesh to give a single cell suspension. After filtering a 200 gauge steel mesh to remove debris and cell clumps, the dispersed cells were washed three times with RPMI-1640 medium (BRL Gibco Co., Betheda, MD) containing 10% heat-inactivated fetal calf serum (BRL Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 X 10^{-5}M 2-mercaptoethanol (2ME) and 10 mM HEPES, and resuspended in the fresh medium at a concentration of 1 X 10^{6} viable cells/ml and used for lymph node (LN) cells.

4. LN cell culture and culture supernatants

To examine blastic activity of LN cells, 100 $\mu\ell$ of suspension containing 1 X 10^{5} cells was dispensed into each well of 96-well flat-bottomed microculture plates. FCS-free RPMI or 50 μg/ml of CII was added another 100 $\mu\ell$ to give a total volume of 200$\mu\ell$, as described previously[11]. These mixture were the incubated at 37℃ in a humidified atmosphere with 5% CO2. After 90 h culture, 37 kBq of ^{3}H-thymidine (^{3}H-TdR: specific activity 740 GBq/mmol; Amersham Co.) was added to each well, and the plate was maintained for another 6 h. Incorporation of 3H-TdR into cells was measured in a Beckman scintillation spectrometer. To prepare culture supernatants, cells were cultured in 24 well plates at a density of 1 x 106 cells in a volume of 1.0 ml. Either 50μg/ml of CII or FCS-free RPMI was added in a volume of 1.0 ml after which the plate was maintained for 48 h at 37℃ in a humidified atmosphere with 5% CO2. Supernatants were collected after pelleting cells by centrifugation at 100 x g for 10 min and stored at -40℃ until used.

5. Cytokine assays

Cytokines of IL-2, IL-6, IFN-γ concentrations in culture supernatants were assayed using rat cytokine ELISA Test kits from R&D Systems (Funakoshi, Co., Ltd., Tokyo, Japan) or BioSource International (CA, USA). Briefly, microplates were coated with 1 μg/ml of anti-cytokine in 50 mM carbonate buffer (pH 9.6) for overnight at 4C, and then the wells were washed 3 times with PBS-0.05% Triton X-100. AFter blocking with 1% BSA in PBS, samples were added to each well and incubated for 2 h at room temperature. The wells were then washed for 5 times. Bound cytokines were detected by Biotinated anti-cytokine and streptoavidin-alkaline phosphatase. After washing, freshly prepared substrate solution (p-nitrophenol phosphate tablet, Sigma Co.) was added to each well. To stop the reaction of color development, 2N-NaOH was added to each well after 20 min. OD was measured at 405 nm. The assay was performed in duplicate accprding to the manufacturer's recommended procedures. The results were expressed as mean±SD (pg/ml) of five individual rats.

6. Arthritis assessment

The clinical symptoms of arthritis in all 4 limbs were evaluated with a visual scoring system. Arthritic lesion of a scale of 0-8: 0 = no change, 2 = swelling and erythema of the digit, 4= mild swelling and erythma of the limb, 6 = gross swelling and erythema of the limb, 8 = gross deformity and inability to use the limb. The arthritis score of each rat was the sum of the scores of each of the 4 limbs, the maximum score being 32. A rat that showed a score of 2or more was regarded to be arthritic. The incidence and day of onset of arthritis were also recorded[12].

7. Measurement of delayed-type hypersensitivity (DTH)

DTH to CII was assessed by the rat ear skin test according to the method described by Cremer et al.[13]. The changes in ear thickness in mm at 48 h were measured after intradermal injection with 10 μg CII dissolved in 10 $\mu\ell$ 50 mM Tris-HCl buffer (pH7.2). The opposite ear was injected with an equal volume of 50 mM Tris-HCl buffer and served as a control. Measurement was made with a dial thickness gauge (Ozaki Sangyou,Tokyo, Japan) and the results were expressed as the difference in thickness between collagen- and buffer-injected ears. Also, the percentage of swelling is calculated by the following formula; (the thickness of footpad after the boosting)/(the thickness of foodpad before the boosting) x 100(%).

8. Analytical methods

Protein contents were determined by a Protein assay kit of Bio-Rad Laboratories (Richmond, CA, USA).

9. Statistics

Results were expressed as means ±SE. Differences were evaluated for significance with the nonparametric Dunnett's multiple comparison test for the arthritic scores, with the Cox-Mantel test for the incidence of arthritis, and the significance of difference between the two groups were evaluated by Student's t test.

III. Results

1. Effect of UD on cytokine production from lymph node cells in response to CII stimulation in vitro.

Continuous i.p injection of UDHA at doses of more than 20 μg/100 g dramatically inhibits the proliferative response to in vitro stimulation of CII.

As shown in Table 1-3, LN cells prepared from immunized rats could secrete much higher levels of T cell cytokines IL-2 (Table 1), IL-6 (Table 2) and IFN-γ (Table 5) in response to CII stimulation. Although treatment of mice with low doses of UD (10 and 15 μ

Table 1. Effect of UD on IL-2 Production of Lymph Node Cells by CII Stimulation in vitro.
(picogram/ml)

IL-2	Dose of UD (μg/100 g)					
	0	5	10	15	20	40
Medium	1.2±0.4	4.6±0.3	3.6±0.4	3.3±0.5	3.0±0.3	2.6±0.2
CII	567.4±35	555.3±46	545.3±45	133.3±45**	10.3±1.2**	9.3±0.9**

The data was expressed as mean ± SD (picogram per ml) of five individual mice. **p<0.01(significant compared with control group).

Table 2. Effect of UD on IL-6 Production of Lymph Node Cells by CII Stimulation in vitro

(picogram/ml)

IL-2	Dose of UD (μg/100 g)					
	0	5	10	15	20	40
Medium	135.5±13.6	145.2±22.3	143.2±23	130.4±21	132.3±13	145.3±13
CII	543.4±46	545.7±32	523.4±23	520.5±55	256.4±21	160.4±16

The data was expressed as mean ± SD (picogram per ml) of five individual mice. * $p< 0.05$ (significant compared with control group).

Table 3. Effect of UD on IFN-γ Production of Lymph Node Cells by CII Stimulation in vitro

(picogram/ml)

IL-2	Dose of UD (μg/100 g)					
	0	5	10	15	20	40
Medium	2.1±0.4	3.4±0.3	2.3±0.3	2.1±0.2	1.2±0.1	0.5±0.2
CII	954.9±65	1213.1±11	1046.2±87	956.1±87	88.3±5.5	42.3±6.5

The data was expressed as mean ± SD (picogram per ml) of five individual mice. * $p< 0.05$ ** $p<0.01$ (significant compared with control group).

g/100 g) did not suppress the ability of LN cells to secrete T cell cytokines, i.p. injection of UD at doses of more than 20 μg/100 g significantly suppressed the production of those cytokines of IL-2, IL-6 and IFN-γ from LN cells.

2. Effect of UD before the primary immunization with CII

Rats were i.p injected with various doses of UD on days -6 and 0 relative to the primary immunization with CII. Pretreatment of rats with UD could inhibit the development of collagen arthritis even when 10-20 μg/100 g/day of the UD was used for pretreatment (Table 4). When doses of 5 and 10 μg UD/100 g mouse were injected, arthritis incidence showed 6/6 and 5/6, respectively, indicating that only 10 μg UD/100 g are effective for arthritis. Interestingly, higher doses that 15 and 20 μg UD/100 g mouse were much effective for arthritis incidence, showing each of 2/6 and 2/6. Similar results were observed

Table 4. Effect of UD Treatment (Days - 6 to 0)on the Collagen Arthritis.

	Dose of UD (μg/100g mouse)				
	5	10	15	20	Saline
Incidence of arthritis	6/6	5/6	2/6	2/6	8/8
Arthritiz index	25.5±2.3	24.2±3.2	21.2±2.4	20.2±2.1	18.4±2.1
Days of onset	25.4±2.2	29.3±2.4	30.2±1.2	31.2±0.21	31.0±2.1
Antibody levels(mg/ml_					
14 days	21.8±2.1	23.1±2.1	27.3±4.2	28.4±3.2	30.2±1.2
24 days	119.3±12.1	122.2±12.2	123.3±14.2	121.2±10.5	123±12.1
DTH*10^2mm)	63.2±2.3	65.4±7.6	70.3±6.3	72.5±3.6	71.2±3.2

The results except for "arthritis incidence" were expressed as mean ± SD. *$p<0.05$; ** $p< 0.01$ (significant compared with control group).

in parameters of Arthritis index, days of arthritis onset, antibody levels (14 days and 24 days) and DTH.

3. Effect of UD treatment from the day of the primary immunization with CII

Rats were 7 daily i.p injected with various doses of UD or saline starting on the day of the primary immunization with CII. Treatment of rats with UD prevented the development of collagen arthritis in a dose-dependent manner (Table 5).

A satisfactory significant prevention of the disease was achieved by treating the rats with 20 μg/100 g/day of the UD, while no clear effects were produced by the treatment with lower dose (10μg/100 g) of the drug. The effects were accompanied by the inhibition of DTH to collagen measured on Day 24 and of anti-CII antibody production on Days 14 and 24.

Doses of UD (15 and 20 μg/100 g) resulted in Incidence of Arthritis of 3/5 and 2/6, respectively. Arthritis indexes of the two doses showed 10.2±1.2 and 6.7±0.5, respectively. On the other hand, days of arthritis onset were 31.3±5.2 and 31.2±2.3, respectively. Similar

results were also observed in antibody levels and DTH of the two doses to be 6.80.6 and 2.40.3 (for 14 days), and 32.52.5 and 14.61.6 (for 24 days), and DTH of 46.4±3.2 and 13.1±1.3 (for 14 days), respectively.

Table 5. Effect of UD Treatment (Days 0 to 7) on Collagen Arthritis.

	Dose of UD (μg/100g)			
	10	15	20	Saline
Incidence of arthritis	6/6	3/5	2/6	8/8
Arthritiz index	20.2±1.4	10.2±1.2	6.7±0.5	21.2±2.3
Days of onset	29.7±3.4	31.3±5.2	31.2±2.3	31.2±2.1
Antibody levels(mg/ml				
14 days	21.2±1.2	7.5±0.5	3.4±0.2	22.1±2.1
24 days	113.4±11.2	32.1±1.2	14.2±1.1	121.2±13.1
DTH*10²mm)	63.1±3.2	46.4±3.2	13.1±1.3	66.2±3.1

The results except for "arthritis incidence" were expressed as mean ± SD. *p<0.05; ** p< 0.01 (significant compared with control group).

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Ⅳ. Discussion

UD is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the UD modify the clinical status of RA are not well understood. In a preliminary study (KS Kim et al., unpublished result), UD inhibited production of IL-2,IL-6 and IFN-γ from macrophags in response to in vivo stimulation with bacterial lipopolysaccharides when the extract was administered into mice once a day for 7 days, suggesting that the UD administered into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. UD treatment, which began concurrently with a booster injection, also significantly suppressed the development of arthritis and immune responses to collagen. The precise mechanisms accounting for these phenomena are not clear, but similar observations were made by Asano et al.[10], who showed that delayed traditional Chinese extract treatment could suppress development of arthritis and of immunity to collagen. It is observed that UD is able to suppress clonal expansion of helper T cells, when it is administered intraperitoneally into rat. Therefore, although the mechanism(s) by which UD exerts suppressive effects on clonal T cell expansion is not well understood, this regimen might theoretically lead to specific clonal depletion and result in inhibition of development of the diseases. An alternative explanation is that the time of a booster injection may still be within the induction phase of arthritis. In this study, we examined the influence of UD on cellular immune responses by using rat CIA, an experimental model for RA. The present results clearly demonstrated that the UD strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro. We examined the effect of the i.p. injection of UD on the development of CIA in rats and on immune responses to CII. We observed that the UD injection has significant reductive effects on the development of CIA in rats at dosages of 100-20μg/100 g/week.

The CIA model has been studied extensively to elucidate pathogenic mechanisms relevant to RA. Similarities between RA and CIA include the fact that susceptibility is linked to specific MHC class II genes[6-7]. Also, the excessive production of several types of cytokines including IFN-γ, IL-2 in the local of affected joints are also observed in these two diseases[13-15]. IFN-γ is generally believed to

play an important role in the pathogenesis of RA by its capacity to induce and enhance the expression of class II MHC antigens on various types of cells[16]. IL-6 and IFN-γ are also thought to be involved in cartilage destruction by stimulating the synthesis of metallproteinase[17-18]. and by inhibiting proteoglycan synthesis[19]. In our present study, it is reasonable to speculate that UD inhibits immune responses, especially cytokine production and antibody formation, and helps to modify the clinical condition of the patient with RA. However, drawing the conclusion that inhibitory action of UD on immune responses is responsible for beneficial effect of the UD on RA, it is necessary to examine the following questions: (1) whether the immune suppressive activity of UD might contribute to the therapeutic mode of action of the UD on RA, and (2) if observed, when the most effective times of starting the treatment with UD are. Therefore, we have used CIA rat and examined a second part of experiment to answer the questions described above.

It is reported that immunization with CII did not induce arthritis or immunity to collagen in congenitally athymic nude rats[8]. It was also reported that arthritis can be passively transferred with immune serum to congenitally athymic nud rats and to cyclosporin A-treated CII tolerant rats. Since the clinical treatment with immunosuppressive agents such as cyclosporin A, FK-506, tripeptide and medicinal plants had a beneficial effect in patients with refractory RA[20-23], UD might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not have serious toxicity, but further studies will be necessary to answer this question.

V. Conclusion

The recommended dose of UD in the management and treatment of rat CIA will be 20㎍/100 g, which is two-firth of human therapeutic dose. However, biochemical and metabolic analysis of the constituents of UD have to be analyzed in further delineating its mechanisms of action in arthritis. The results explain that UD is effective for the treatment of osteoarthritis in patients.

VI. References

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