원 저

Anti-inflammatory Effects and Mechanisms of *Ulmus Davidiana* Planch(Ulmaceae) in Collagen-induced Arthritis Rats

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국문초록

쥐의 콜라겐 유도 관절염에 대한 유근피의 효과 및 기전

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목적 : 쥐의 콜라겐 유도 관절염에 대한 유근피 추출액의 면역 반응 효과 및 그 기전을 살펴보고자 하였다.

방법 : 유근피 추출액의 면역 반응을 관찰하기 위하여 콜라겐 유도 관절염 쥐가 사용되었다. 실험에 쓰인 쥐 뒷다리의 부종 용적은 volume meter로 측정하였고, lymphocyte 증식, IL-1, IL-2 및 TNF-α 레벨은 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide(MTT) assay에 의해 측정하였다. 활막세포의 cAMP 레벨은 경쟁적 단백 결합검사(CPBA)를 통하여 측정하였다. 2형 콜라겐에 대한 항체는 효소면역 협착검사법(ELISA)을 반복 사용하여 측정하였다.

결과 : 실험에서 유근피 추출액(20, 80, 150㎎/㎏, ig × 7days)의 시술은 면역 반응을 억제하고 콜라겐 유도 관절염 쥐의 체중과 면역 기관의 무게를 유지하였다. 콜라겐 유도 관절염 쥐에서 림프구의 증식과 IL-2의 생산은 복막의 대식세포 및 활막세포의 IL-1, TNF-α와 함께 증가하였고, 유근피 추출액 (20, 80, 150㎎/㎏, ig × 7days)의 시술은 이러한 변화를 유의성 있게 감소시켰다. 0.5, 2.5, 12.5, 62.5, 125㎎/ℓ 농도에서의 유근피 추출액은 활막세포의 cAMP 레벨을 증가시킨데 반해 콜라겐 유도 관절염 쥐에서의 시험관 실험 결과에서는 감소시켰다. 유근피 추출액은 2형 콜라겐 항체의 농도에 대하여는 효과가 없었다.

· Acceptance : 2007년 3월 3일 · Adjustment : 2007년 3월 16일 · Adoption : 2007년 3월 16일
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결론 : 유근피 추출액은 항염증 작용과 면역조절 작용을 갖고 있고, 활막세포의 G protein-AC-cAMP transmembrane signal transduction 형질 도입 신호에 의한 콜라겐 유도 관절염 쥐의 치료 효과를 가지고 있는 것으로 여겨진다.

핵심단어 : *Ulmus davidiana* Planch (Ulmaceae), Collagen-induced arthritis rats, Cyclic AMP, Synoviocytes

I. Introduction

Rheumatoid arthritis(RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints, with degeneration of cartilage and bone erosion. The main pathology of the affected synovial tissues consists of hyperplasia and subintimal infiltration of T and B lymphocytes. RA is classically treated with anti-inflammatory and immunosuppressive drugs, whose side effects are well known[1].

Collagen-induced arthritis(CIA) is a well-known experimental model resembling human RA. CIA resembles RA in a number of pathological, histological, and immunological aspects[2]. Features of CIA include chronic synovitis, including inflammatory cell infiltration, pannus formation, destruction of cartilage, and bone erosion. The immune mechanisms that include both humoral and cellular immunity to CII have been implicated in the pathogenesis of the disease[3].

There are several reports that traditional korean medicines, including bee venom acupuncture[4], elctroacupuncture[5], deer antler aqua-acupuncture[6] and *Ulmus davidiana* Planch(Ulmaceae, UD) water extracts[7], have anti-inflammatory effects on CIA rats.

UD is a deciduous tree, which is widely distributed in Korea and has have long been known to have anti-inflammatory and protective effects on damaged tissue, inflammation and bone resorption[7-12]. Especially, UD has been proved that administration of UD has therapeutic effects on CIA including protection of cartilage and RA for a potential therapy[7].

To refer to a similar research, a study with glucosides from chaenomeles speciosa(GCS) of Chinese medicine on CIA rats was founded to be similar with current research[13], showed the quite resembling effects on inflammatory and immune responses. They have assessed lymphocyte proliferation, interleukin-1, interleukin-2, TNF-a level levels. cAMP level in synoviocytes and mRNA expression of Gi, Gs, and TNF-a of synoviocytes in CIA rats were also analyzed. The results clearly showed that there is a marked secondary inflammatory response in CIA model, which accompanied with the decrease of body weight and the weight of immune organs simultaneously. The administration of GCS(30, 60, 120㎎/㎏) for 7days inhibited the inflammatory response and restored body weight and the weight of immune organs of CIA rats. Lymphocyte proliferation and IL-2 production of CIA rats increases, together with IL-1 and TNF-a in peritoneal macrophages and synoviocytes.

Previous studies showed that UD possesses anti-inflammatory and an-algesic properties. However, it is unknown whether UD exerts its effect on the chronic autoimmune diseases such as RA. The present study was therefore designed to investigate the effects of UD on CIA rat and its relative mechanisms by which UD affects the process of CIA, especially synoviocyte function.

The goal of this study was to investigate the effects of UD in the pathogenesis of arthritic disease by interfering with cytokine-signaling pathways. This study also aimed to develop a new therapeutic strategy for the treatment of RA by blockade of intracellular cytokine-signaling pathways.

Ⅱ. Experiments

1. Materials

1) Animals

Male Sprague-Dawley(SD) rats (180±20g, Gradell, Certificate No. 006), 6weeks old were used in this study. Rats were obtained from Genetic Resource Center, Research Institute of Bioscience and Biotechnology, Daejeon, Korea. During the experimental period, they were kept under standard laboratory conditions, and tap water and commercially available food(CE-2, CLEA, Tokyo, Japan; calcium content 1.18/100g; phosphorus content 1.09/100g; vitamin D_3 content 250 10 U/100g) were given freely. The lighting duration in the breeding room was 12h(7:00 am to 7:00 pm). The room temperature was 24°C. The experiment was approved by Committee of Laboratory Animals, Faculty of Medicine and Oriental Medicine, Dongguk University, Korea.

2) Plant material

UD was purchased from a market specializing in herbs(Kyungju herb market, Kyungju, Korea) 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.

3) Chemical and drugs

Chicken type Ⅱ collagen was obtained from Institute Jacques Boy(Reims, France). Lipopolysaccharide(LPS, Escherichia coli 0111:B4), concanavalinA(ConA), 3-(4,5-2dimethylthiazal-2yl) 2,5-diphenyltetrazoliumbromide(MTT), and all chemicals were purchased from Sigma(St. Louis, MO, USA). PBS was obtained from Eurobio(Les Ulis, France). The RPMI-1640 medium was supplemented with Hepes, 10mM L-glutamine, 2 mM 2-mercaptoethanol, 50M penicillin sodium, 100 kU/ℓ; streptomycin, 100㎎/ℓ; and 10% new born bovine serum and was adjusted pH to 7.2. The 96-well flat-bottom Maxisorb microtiter plates were obtained from Nunc(Life Technologies, Cergy Pontoise, France). 50mM stock solutions of indomethacin(Sigma) and 10 mM stock solution of celecoxib[SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazolyl]- benzenes- ulfonamide; Searle Research and Development, St. Louis, MO, USA] were prepared with DMSO(Sigma) and diluted with culture medium to final concentrations of 5-400μM. Control cells received DMSO(0.25%) or culture medium only.

2. Methods

1) Preparation of UD herbal extract solution (UDHES) and fractions

The herb had a moisture content of <10% by weight, and was air-dried. Air-dried barks(70g dry weight) were mixed, minced with a grinder, and extracted by storing in 1ℓ of boiling water for 3hours. The supernatant was filtered with 10 ㎛ cartridge paper and ethanol was removed by rotary evaporation(Eyela, Tokyo, Japan), and concentrated extracts were freeze-dried. This process generally produced 15g of brown powder.

The powder form of the extract was dissolved in medium to 20㎎/㎖, vortexed at room temperature for 1 minute, and incubated at 37°C for 1hour while rotating before use. This solution was centrifuged at 5,000rpm for 10minutes to remove any insoluble ingredients. The supernatant was passed through a 0.22㎛ filter for sterilization and diluted with culture medium to final concentrations of 1-40㎍/㎖ UD herbal extract solution (UDHES). A voucher specimen has been deposited at the Kyungju Oriental Medical Hospital, Dongguk University, Kyungju city, Kyungbuk, Korea under acquisition number UD-13.

2) Induction of CIA and UD treatment

CIA was induced by immunizing rat with native Chicken type II collagen(CII) that had been dissolved overnight at 4°C in 0.1M acetic acid (4 ㎎/㎖) and emulsified with an equal volume of IFA(Difco Laboratories, Detroit, MI, USA). Rats were injected intradermal(i.d.) twice with 200㎍ of emulsified CII. The first injection was made in a hind metatarsal footpad; the second, 7days later, into the proximal one-third of the tail[14].

Before the onset of arthritis, animals were divided into five groups randomly. On the 10 after immunization, rats were given intragastrically UD (20, 80, 150㎎/㎏/day) and continued through days 10 to 20. For the normal and CIA model rats were given an equal volume of distilled water.

3) Evaluation of CIA

CIA was monitored and evaluated throughout the experiment measuring the hind-paw edema by two observers blinded to treatment from the onset. To evaluate quantitatively the severity of the arthritis, a scoring system was used that correlates the arthritis severity with joint size. Rat paws were scored for arthritis, as previously described[15], inflammation of the four paws was graded from 0 to 4: grade 0, paws with no swelling and focal redness; grade 1, paws with swelling of finger joints; grade 2, paws with mild swelling of ankle or wrist joints; grade 3, paws with severe inflammation of the entire paws; and grade 4, paws with deformity or ankylosis. Each paw was graded and the four scores were totaled so that the maximum possible score per rat was 16.

4) Lymphocytes proliferation assay

Rats were sacrificed by cervical dislocation. Thymus and spleen were removed in sterile condition. Thymocytes and splenocytes were isolated by routine method. Then, the cells were suspended in RPMI-1640 medium at a concentration of 1×10^{10}cell/ℓ. Cell suspension (100ℓ) and 100 l ConA(with final concentration of 5㎎/ℓ) or 100ℓ LPS(with final concentration of 4 ㎎/ℓ) were seeded to 96-well flat-bottomed culture plate, respectively. The cultures were incubated at 37°C, 5% CO_2 for 48 hours. A 10㎕ sample of MTT(5g/ℓ) was added to each well, oscillated for 1minute on oscillator at 37°C and 5% CO_2 for 2hours continuously. After incubation, the cultures were centrifuged (760 × g, 10minute). The supernatants were aspired, 120㎕ of isopropanol(containing HCl 0.04mol/ℓ) was added to each well and oscillated for 30 seconds again. The absorbance (*A*) was measured on EJ301 ELISA Microwell Reader at 570㎚. The results were described as the average of *A*.

5) Measurement of IL-1 activity

IL-1 activity was measured by ConA-induced thymocyte proliferation assay. Exudated macrophages of rats were prepared by the intraperitoneal injection of 2.5% glycogen solution. After 4 days, peritoneal exudated cells were collected with RPMI-1640 media. The cells were washed three times in RPMI-1640 media. The cell suspensions were adjusted to 2×10^9cell/ℓ in RPMI-1640 media containing 10% FCS and dispensed at 1㎖/well in 24-well plates. After incubation for 2 hours at 37°C in a humidified 5% CO_2 atmosphere, nonadherent cells were removed by washing twice with RPMI-1640. Then, 100㎕ LPS(4㎎/ℓ) plus 0.9㎖ RPMI-1640 medium were added at 37°C in air with 5% CO_2 for 48hours, The cultures were centrifuged (1000 × g, 5minutes) and the sample supernatants containing IL-1 were collected and stored at -20°C until assay for IL-1 activity.

50㎕ of suspension of thymocytes(2×10^{10}cell/ℓ) taken from rats was distributed over a flat-bottomed 96-well microtiter plate. Then, 100 ㎕ sample supernatants of peritoneal macrophages (PMΦ) containing IL-1 and 50㎕ ConA(5㎎/ℓ) were added incubated for 48 hours at 37 °C in air with 5% CO_2(similar to *Lymphocytes proliferation MTT assay*).

6) Measurement of TNF-α activity

Exudated macrophages of rats were obtained by the intraperitoneal injection of 2.5% glycogen solution. After 4days, peritoneal exudated cells were collected with RPMI-1640 media. The cells were washed 3times in RPMI-1640 media. The cells suspended in RPMI-1640 media containing 5% heat-inactivated serum derived from normal rats were seeded to 24-well culture plates 1×10^9 cell/ℓ and incubated at 37°C, 5% CO_2 for 4 hours. Nonadherent cells were removed by washing twice with RPMI-1640. Then, 100㎕ LPS (4㎎/ℓ) plus 0.9㎖ RPMI-1640 medium were added at 37°C in air with 5% CO_2 for 48 hours. The cultures were centrifuged (1000 × g, 5 minutes) and the sample supernatants containing TNF-α were collected and stored at -20°C until assay.

The base of this assay is the quantitation of the cytotoxic activity of TNF-α on L929 cells in the presence of actinomycin D[16]. The mitochondrial reduction of MTT to formazan was determined as an indicator of L929 cell viability[17]. Briefly, L929 cells were seeded, at a density of 2×10^4 cells/well, into a 96-well microtiter plate. After incubation for 24hours at 37°C in a humidified atmosphere with 5% CO_2, the medium in the wells was replaced with fresh medium containing actinomycin D at a final concentration of 1㎍/㎖. After incubation with different concentrations of TNF-α at 37°C for 48hours, 10ℓ MTT(5㎎/㎖ in PBS, pH 7.2) was added to each well. After an additional 4-h incubation at 37°C, 150㎕ of 0.04 M HCl in isopropanol was added to each well. Once the dark blue formazan had been dissolved, the absorbance of each well was measured with a Titertek Multiskan MCC 340 microplate reader using a test wavelength of 570nm and a reference wavelength of 630㎚. Cytotoxicity by MTT conversion at each concentration of TNF-α was compared with untreated(control) cells(shown as 100%). The ID_{50} value was taken as the concentration of drug required to produce a 50% inhibition of cell growth. This was calculated from a logarithmic regression curve of the results for at least five separate experiments.

7) Measurement of IL-2 activity

Thymocytes of rats were collected by routine method after removing in sterile condition. Then the cells were suspended in RPMI-1640 medium at a concentration of 5×10^{10}cell/l. A 100㎕ sample of suspension, 100㎕ of ConA with final concentration of 5㎎/ℓ, and 800㎕ RPMI-1640 medium were added to each well of 24-well culture plate, respectively, making the final volume 1㎖. The cultures were centrifuged(500 × g, 10 minutes) after incubation at 37 °C, 5% CO_2 for 48 hours. The supernatants were collected, and reserved at -20 °C.

The activated murine splenocytes proliferation was used at the estimation of IL-2. Spleen cells of rats were suspended at a concentration of 2×10^9cell/l, added with 5㎎/ℓ ConA, incubated at 37°C, 5% CO_2 for 4 days and washed three times with 5% bovine-Hank's. Suspension of activated rats splenocytes was adjusted to 1×10^9cells/l with RPMI-1640 medium. A 100ℓ sample of suspension and 100㎕ of IL-2 supernatants were added to each well of 96-well culture plate, respectively, incubated at 37°C, 5% CO_2 for 24 hours(similar to Lymphocytes proliferation MTT assay).

8) Preparation and culture of synovial cells

As described method[18], synovial cells were prepared by collagenase and DNase digestion of small minced membranes as described[18], with minor modifications. Synovial tissues were digested in RPMI-1640 medium containing 5% fetal bovine serum(FBS; JRH Biosciences, Lenexa, KS, USA), 1㎎/㎖ collagenase A(Roche Diagnostics, Sandhoferstr, Germany), and 0.15㎎/㎖ DNase I (Roche) for 2hours at 37°C, 5% CO_2. After incubation, the tissue was pipetted through sterile 108㎛² 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 (Sumitomo Bakelite, Tokyo, Japan) at 5 × 10^5cells/well in RPMI-1640 containing 5㎎/ℓ LPS, 100IU/㎖ penicillin, and 100㎍/㎖ streptomycin, at 37°C, 5% CO_2. Supernatants were harvested at 48 hours and stored at -20°C until measurement for the production of IL-1 and TNF-α.

9) Synoviocytes proliferation assay

According to the above method synoviocytes were isolated. Then the cells were suspended in RPMI-1640 medium with 15% FBS at a concentration of 2 × 10^9cell/ℓ. The cell suspension of 100㎕ were added to 96-well flat-bottomed culture plate and incubated at 37°C, 5% CO_2 for 24hours. After cells adherent, the cultures were replaced by RPMI-1640 medium without bovine serum and incubated at 37°C, 5% CO_2 for 24 hours. Aspiring RPMI-1640 medium without bovine serum and adding 100㎕ RPMI-1640 medium with 15% FBS, the cultures were incubated at 37°C, 5% CO_2 for 66 hours (then similar to *Lymphocytes proliferation MTT assay*).

10) Assay for cAMP of synoviocytes

Competitive protein binding assay (CPBA) was done to evaluate cAMP according to the procedures described by Lopez-Gonzalez et al.[19]. Rats were sacrificed by cervical dislocation. Synoviocytes were isolated according to the above method. Then the cells were suspended in RPMI-1640 medium at a density of 1 × 10^9cell/l and centrifuged (1500×g, 10minutes). The supernatant was quickly removed and cell precipitate were boiled at 100°C for 5min and reserved at -20°C. Then the cAMP were determined using a cAMP kit (Yamasa, Chousi, Japan) following manufacturer's instructions.

11) Measurement of anti-CII antibodies

Under ether anesthesia blood was collected from the rats by cardiac puncture after the serum level of IgG antibodies to CII was determined by the enzyme-linked immunosorbent assay(ELISA) on days 16 and 35. To be breif, a 100ℓ sample of serum(1 : 4,000) in phosphate-buffered saline (PBS^-; Nissui Pharmaceutical, Tokyo, Japan) containing 0.05% Tween-20(Nacalai Tesque, Kyoto, Japan) and 1% bovine serum albumin(BSA; Fraction V, Boehringer Mannheim) was added to the wells of a 96-well microtiter plate (Nunc Immunplate I, Intermed, Roskilde, Denmark) that had been coated with CII overnight at 4°C. The plate was incubated for 2hours at room temperature then after the washing of the wells with PBS^- containing 0.05% Tween-20, an addition was made of 100ℓ of peroxidase-labeled goat anti-mouse IgG (1 : 600; Organon Teknika N.V. Cappel Products, West Chester, PA, USA) in PBS^- containing 0.05% Tween-20 and 1% BSA. After incubation for 1hour at room temperature, the wells were washed with PBS^- containing 0.05% Tween-20, after which 100㎕ of 0.05% phenylenediamine containing 0.017% H_2O_2 solution was added. After incubation for 1 hour at room temperature, the reaction was stopped by the addition of 50㎕ of 4 N H_2SO_4. Color was allowed to develop for 20minutes, and then the optical density was measured at 490㎚ with a microplate photometer(MTP-32, Corona Electric Ibaragi, Japan).

12) Measurement of delayed-type hypersensitivity (DTH) reaction to CII

The changes in ear thickness in millimeters(Δ㎜) were measured 24 hour after I.d. injection of 20㎍ of CII dissolved in 0.02㎖ of PBS on day 36. DTH was regarded as its changes in ear thickness. The opposite ear was injected with an equal volume of PBS and served as a control. Measurements were made with a digital micrometer(M-30, Sony Magnescale, Tokyo, Japan) under ether anesthesia and were expressed as the difference in thickness between collage- and

PBS-injected ears.

13) Histological examination

The removed legs and hind paws of rats were fixed with 10% paraformaldehyde in PBS, and then decalcified for 10days with EDTA and embedded in paraffin for histologic analysis. The paraffin sections were stained with hematoxylin and eosin. The slides were evaluated histologically with optical microscope(Olympus BX 50, Olympus Optical, Co. Japan) by two independent observers, and the gradation of arthritis was scored from 0 to 4 according to the intensity of lining layer hyperplasia, mononuclear cell infiltration, and pannus formation, as described previously[18]: 0, normal ankle joint; 1, normal synovium with occasional mononuclear cells; 2, definite arthritis, a few layers of flat to rounded synovial lining cells and scattered mononuclear cells; 3, clear hyperplasia of the synovium with three or more layers of loosely arranged lining cells and dense infiltration with mononuclear cells; 4, severe synovitis with pannus and erosions of articular cartilage and subchondral bone.

14) Statistical analysis

Values in tables and figures are expressed as means and standard deviation of the mean if not otherwise indicated. The analysis of variance (ANOVA) and Student's t-test are used in the program SPSS(SPSS SigmaStat Software Products, Chicago, IL, USA) to determine significant differences between groups. Values of p less than 0.05 ($p<0.05$) were considered to be significant.

Ⅲ. Results

1. Effect of UDHES on secondary arthritis in CIA rats

In this model, there was a marked inflammatory response. The onset of secondary arthritis appeared on about day 15 after injection of CII, with a peak onset at day 20. The CIA rats was administrated UDHES (20, 80, 150㎎/kg/day, ig×10 days, days 10-20) which inhibited the inflammatory responses (Table 1, 2).

Table 1. Effect of UDHES on Secondary Arthritis in CIA Rats

	Days after immunization					
	0	10	15	20	25	30
Paw swelling(Δmℓ)						
Normal control	0	0.03±0.001	0.04±0.001	0.05±0.001	0.08±0.001	0.11±0.001
CIA rat	0	0.04±0.002	1.1±0.2**	1.3±0.2**	1.0±0.1**	0.6±0.1**
UDHES 20㎎/kg	0	0.04±0.002	1.0±0.1	1.2±0.2	0.9±0.1	0.5±0.02#
UDHES 80㎎/kg	0	0.04±0.002	0.9±0.1#	1.1±0.1#	0.8±0.1#	0.5±0.02#
UDHES 150㎎/kg	0	0.04±0.002	0.8±0.1##	0.9±0.1##	0.6±0.1##	0.3±0.02##

Each 5-day intervals, the degree of swelling of non-injected hind paw was measured(in Δmℓ). UDHES significantly suppressed hind paw swelling from days 15 to 30 post-immunization. There is no increase at hind paw volume over time in normal rats. $\bar{x} \pm s$, n=6. **p<0.01 vs. Normal group, #p<0.05, ##p<0.01 vs. CIA model.

Table 2. Effect of UDHES on Polyarthritis of CIA Rats

	Days after immunization					
	0	10	15	20	25	30
Arthritis score						
Normal control	0	0.01±0.001	0.01±0.001	0.01±0.001	0.01±0.001	0.11±0.001
CIA rat	0	1.4±0.2	4.4±0.4**	5.2±0.6**	4.3±0.3**	4.0±0.3**
UDHES 20㎎/kg	0	1.4±0.2	4.0±0.4	4.5±0.5#	3.6±0.3#	3.1±0.2#
UDHES 80㎎/kg	0	1.3±0.1	3.6±0.3#	4.0±0.4#	3.0±0.1##	3.0±0.02##
UDHES 150㎎/kg	0	1.3±0.1	3.1±0.2##	3.4±0.2##	3.2±0.1##	3.0±0.02##

Significant increase was measured in the arthritic score between days 15 and 30, $p<0.01$. UDHES significantly suppressed the arthritic score between days 15 and 30. $\bar{x} \pm s$, n=7. **p<0.01 vs. Normal group, #p<0.05, ##p<0.01 vs. CIA model.

2. Effect of UDHES on body weight and the weight of immune organs of CIA rats

The body weight comparison were studied by the rate of body weight gain between CII-immunized and non-immunized rats. Beginning on day 15, the CII-immunized rats gained significantly less weight than the normal group, and this trend continued through day 30. After administration of UDHES, the CIA group showed a significant weight gain. The index of thymus and spleen of CIA rats were determined at day 31 after immunization. There was a decrease of thymus and spleen in CIA rats. The administration of UDHES(20, 80, 150㎎/kg, ig × 10days) increased significantly the weight of immune organs of CIA rats(Table 3, 4).

Table 3. Effect of UDHES on Body Weight of CIA Rats

	Days after immunization					
	0	10	15	20	25	30
Increase of body weight(Δg)						
Normal control	0	10±1.61	15±2.0	30±3.6	50±6.6	65±6.0
CIA	0	10±1.2	8±0.6**	15±1.4**	23±3.1**	28±3.0**
UDHES 20㎎/kg	0	9.5±0.8	13±1.3	27±3.6#	46±5.0#	57±5.2#
UDHES 80㎎/kg	0	9.0±0.7	13±1.5#	25±3.3##	44±4.0##	56±5.5##
UDHES 150㎎/kg	0	9.0±0.7	12±1.1#	24±2.7##	43±2.3##	55±6.2##

The type II collagen(CII)-immunized rats gained significantly less weight than the normal group beginning on day 20, a trend continued through day 30. UDHES treatment positively increased the weight gain of the CII-immunized rats. $\bar{x} \pm s$, n=10. **p<0.01 vs. Normal group, #p<0.05, ##p<0.01 vs. CIA model.

Table 4. Effect of UDHES on Index of Thymus and Spleen in CIA Rats($\bar{x} \pm s$, n=7)

Group	Dose (mg/kg/day)	Index of immune organs(%)	
		Thymus	Spleen
Normal control		0.11±0.01	0.20±0.02
CIA		0.08±0.01**	0.18±0.03**
CIA+UDHES	20	0.10±0.02#	0.20±0.03#
CIA+UDHES	80	0.11±0.02##	0.21±0.03##
CIA+UDHES	150	0.12±0.03##	0.22±0.03##

**p <0.01 vs. Normal group.
#P<0.05 vs. CIA model.
P<0.01 vs. CIA model.

3. Effect of UDHES on lymphocytes proliferation and production of IL-2 in CIA rats

The lymphocytes proliferation and IL-2 production of CIA rats were determined at day 31 after immunization. There were an increase of ConA-induced and LPS-induced lymphocyte proliferation and IL-2 production in CIA rats. The administration of UDHES (20, 80, 150mg/kg, ig×10 days) decreased significantly the lymphocyte proliferation and IL-2 production, except UDHES 20 mg/kg in LPS-induced lymphocyte proliferation (Table 5).

Table 5. Effect of UDHES on Proliferation of Lymphocytes and Production of IL-2 in CIA Rats($\bar{x} \pm s$, n=4)

Group	Dose (mg/kg/day)	ConA-induced(A)	LPS-induced(A)	IL-2(A)
Normal control		0.59±0.05	0.44±0.03	0.48±0.05
CIA		0.72±0.06**	0.45±0.04*	0.68±0.07**
CIA+UDHES	20	0.64±0.05#	0.49±0.05	0.51±0.04#
CIA+UDHES	80	0.63±0.06#	0.37±0.05#	0.41±0.05##
CIA+UDHES	150	0.55±0.06##	0.28±0.03##	0.43±0.04##

**p <0.01 vs. Normal group.
#P<0.05 vs. CIA model.
P<0.01 vs. CIA model.

4. Effect of UDHES on synoviocytes proliferation of CIA rats

The synoviocytes proliferation of CIA rats was determined at day 31 after immunization. It was found that there were increases of synoviocytes proliferation in CIA rats. The administration of UDHES(20, 80, 150mg/kg, ig×10days) decreased significantly the synoviocytes proliferation(Table 6).

5. Effect of UDHES on IL-1 and TNF-α production by peritoneal macrophages and synoviocytes in CIA rats

The cytokines of CIA rats were determined on day 31 after immunization. IL-1 and TNF-α production from peritoneal macrophages (PM) and synoviocytes increase compared with normal group. The administration of UDHES(20, 80, 150

Table 6. Effect of UDHES on Synoviocytes Proliferation of CIA Rats($\bar{x} \pm s$, n=4)

Group	Dose(㎎/kg/day)	Synoviocytes proliferation(A)
Normal control		0.20±0.02
CIA		0.28±0.03**
CIA+UDHES	20	0.25±0.04#
CIA+UDHES	80	0.23±0.02##
CIA+UDHES	150	0.22±0.03##

**p <0.01 vs. Normal group.
#P<0.05 vs. CIA model.
P<0.01 vs. CIA model.

㎎/kg, ig×10days) decreased significantly the production of IL-1 and TNF-α from peritoneal macrophages and synoviocytes(Table 7).

6. Effect of UDHES on cAMP optical density of synoviocytes in CIA rats in vitro

On the basis of the above results, further studies for the mechanisms of UDHES on cAMP level of synoviocytes were done in vitro. It showed that the cAMP level of synoviocytes was lower in CIA rats than that in normal. UDHES at the concentration of 1.0, 5.0, 20.0, 50.0, 100, 200㎍/㎖ increased above decreased cAMP levels of synoviocytes in CIA rats(Table 8).

7. Effect of UDHES on anti-CII antibodies

A significant elevation of the serum antibodies to CII was found in CIA rats. UDHES had no effect on the concentration of anti-CII antibodies (Table 9).

8. Effect of UDHES on delayed-type hypersensitivity (DTH) reaction to CII

It showed that the ear thickness was significantly increased in CIA rats than that in normal 24h after challenge with an intradermal injection of CII. UDHES(80, 150㎎/kg, ig×10 days) suppressed significantly the delayed-type skin reaction to CII(Table 10).

9. Effect of UDHES on histology of CIA rats

Histological features were observed on day 31 after immunization. Rats were satisfied and subjected to histological observation. In normal rats, it showed normal cartilage-bone inflammation. Whereas in CIA rats treated with vehicle, it showed marked mononuclear cell infiltration with cartilage-bone destruction. Synovial lining cell hyperplasia was still observed in CIA rats treated with UDHES 20㎎/kg(grade 3), but only limited synovial lining cell hyperplasia was detected in CIA rats treated with UDHES 50㎎/kg(grade 2). In CIA rats treated with UDHES 80㎎/kg(grade 1), only limited synovial lining cell hyperplasia was observed.

Table 7. Effect of UDHES on IL-1 and TNF-α Production of Peritoneal Macrophages and Synoviocytes in CIA Rats ($\bar{x} \pm s$, n=4)

Group	Dose (㎎/㎏/day)	IL-1 (A)		TNF-α activity (U/㎖)	
		PMϕ	Synovial Cells	PMϕ	Synovial Cells
Normal control		0.480±0.043	0.556±0.047	118.6±16.2	46.5±6.0
CIA		0.712±0.062**	0.783±0.047**	231.5±32.3**	73.6±5.3**
CIA+UDHES	20	0.523±0.047##	0.574±0.362##	146.6±25.6#	52.7±5.2#
CIA+UDHES	80	0.467±0.037##	0.531±0.543##	123.2±14.4##	47.5±4.4##
CIA+UDHES	150	0.450±0.043##	0.468±0.426##	113.4±12.1##	43.2±3.6##

**p <0.01 vs. Normal group.
#P<0.05 vs. CIA model.
P<0.01 vs. CIA model.

Table 8. Effect of UDHES on cAMP Optical Density of Synoviocytes in CIA Rats in vitro ($\bar{x} \pm s$,n=4)

Group	Concentration Dose(㎎/ℓ)	cAMP (pmol/106 cell)
Normal control		8.02±0.56
CIA		2.83±0.31**
CIA+UDHES	0.5	3.83±0.33#
CIA+UDHES	1.0	4.66±0.63##
CIA+UDHES	2.0	5.65 ±0.46##
CIA+UDHES	5.0	6.23 ±0.53##
CIA+UDHES	10.0	6.83 ±0.67##
CIA+UDHES	20.0	7.84 ±0.84##
CIA+UDHES	50.0	7.95 ±0.04#

**p <0.01 vs. Normal group.
#P<0.05 vs. CIA cells.
P<0.01 vs. CIA cells.

Table 9. Effect of UDHES on The Optical Density of serum anti-CII antibodies in CIA rats ($\bar{x} \pm s$, n=10)

Group	Dose (㎎/㎏/day)	Days after immunization (A, 490 nm)	
		day 15	day 30
Normal control		0	0
CIA		0.680±0.051**	1.018±0.047**
CIA+UDHES	20	0.681±0.042	0.994±0.034
CIA+UDHES	80	0.673±0.032	0.990±0.066
CIA+UDHES	150	0.671±0.043	0.922±0.052

**p <0.01 vs. Normal group.

Table 10. Effect of UDHES on delayed-type skin reaction to CII in CIA rats($\bar{x} \pm s$, n=10)

Group	Dose(mg/kg/day)	Ear thickness(mm)
Normal control		0.048±0.013
CIA		0.802±0.093**
CIA+UDHES	20	0.772±0.054
CIA+UDHES	80	0.690±0.062#
CIA+UDHES	150	0.542±0.034##

**p <0.01 vs. Normal group.
#p<0.05 vs. CIA cells.
##p<0.01 vs. CIA cells.

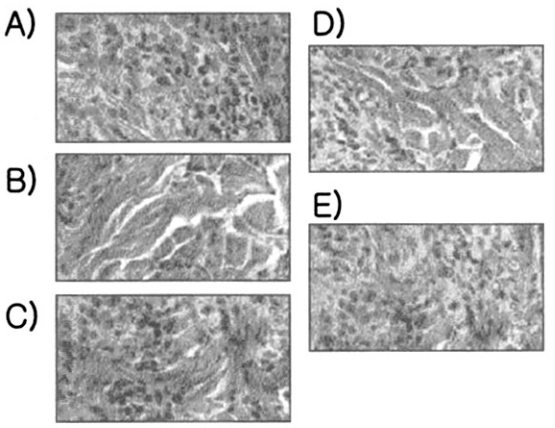

Fig. 1. Histological Illustrations of Cartilage Bone in CIA Rats

A) Normal cartilage-bone inflammation (original magnification ×300).
B) In CIA rats treated with vehicle. It shows marked mononuclear cell infiltration with cartilage-bone destruction (original magnification ×300).
C) In CIA rats treated with UDHES 20mg/kg(grade 3); still synovial lining cell hyperplasia was detected (original magnification ×300).
D) In CIA rats treated with UDHES 50mg/kg(grade 2); only limited synovial lining cell hyperplasia was detected (original magnification ×300).
E) In CIA rats treated with UDHES 80mg/kg(grade 1); only limited synovial lining cell hyperplasia was detected (original magnification ×300).

Ⅳ. Discussion and Conclusion

Joint swelling, arthrodynia and deformity were main clinical symptoms of RA. Rat adjuvant arthritis (AA) induced by CFA had similar characteristics to RA in the aspects of histology and immunology. The present study demonstrated that UD markedly inhibited joint swelling and pain, as well as down-regulated the index of polyarthritis in AA rats. This suggested that UD might be effective on chronic autoimmune disease such as RA. This provided the further consideration that UD might be a new class of effective anti-inflammatory agents. The main pathological changes of RA included synovitis and pannus formation, which lead to cartilage erosion and articular destruction. Synoviocytes were the ultimate effectual cells of

pathologic change[20]. In our study, UD ameliorate the secondary inflammatory reaction of AA via influencing secretory function of activated synoviocytes.

Many popular uses of plant extracts against chronic inflammatory diseases have been stimulating the investigation of plant extracts on different arthritic experimental models. Clinical benefits for treating experimental arthritis have been demonstrated with UD of these plant extracts. Although several studies demonstrated the anti-inflammatory and anti-arthritic effect of UD including release several cytokines, in this present study I have clarified that UDHES could modulate the cellular and humoral immunity in CIA rats. Also, I researched G protein coupled AC-cAMP signal pathway for the immunoregulation of UD.

UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue[12]. The research has shown that UD has actions such as anti-inflammation, analgesia, and immunoregulation. UD possesses the inhibition on acute inflammation of rat induced by adjuvant-carrageenan and inhibits primary and secondary inflammation on adjuvant-induced arthritis[7].

Because UD has a strong anti-inflammatory effect, it was hypothesized that its anti-inflammatory activity may derive from inhibition of cyclooxygenase-2 (COX-2) pathway. As a part of the search for new biologically active substances from traditional medicines, the author evaluated whether extracts of UD stem barks could modulate the induction of rheumatoid arthritis (RA) in rats.

UD has been shown to have a broad spectrum of biological activities, including anti-inflammatory and anticancer activity based on its long history in clinical applications. UD is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional oriental medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support[21,22]. Although the barks of U. davidiana stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-inflammatory action in vivo.

Recently, glycoprotein of *Ulmus davidiana* Nakai was shown to have strong scavenging activities against oxygen free radicals. The glycoprotein has inhibitory effects on protein kinase C alpha (PKCα) translocation, nuclear factor-kappa B (NF-κB) DNA binding activity, nitric oxide (NO) production, and apoptosis in 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated NIH/3T3 cells. Interestingly however, it could not regulate the DNA binding activity of AP-1. Therefore, UDN glycoprotein, a natural anti-oxidant, was believed is a potential modulator of apoptotic signal pathways in NIH/3T3 cells[10]. Four lignan xylosides and two neolignan glycosides were isolated from the stem and root barks of *Ulmus davidiana* var. japonica. Their structures were identified as lyoniside, nudiposide, 5′-methoxyisolariciresinol-9′-O-beta-D-xylopyranoside, isolariciresinol-9′-O-1-D-xylopyranoside, rel-trans-dihydrodehydroconiferyl alcohol 4′-O-alpha-L-rhamnopyranoside and icariside E3 by comparison of their spectral data with those reported in the literatures, respectively[23]. Investigation of the constituents of the stem and root barks of *Ulmus davidiana* var. japonica resulted in the isolation of five new triterpene esters named ulmicin A-E. Their structures were determined to be 3beta, 11alpha, 15alpha-trihydroxylup-20 (29)-ene-11-(3′-methoxy-4′-hydroxybenzoyl ester), 3beta,11alpha,15alpha-trihydroxylup-20 (29)-ene-11-(4′-hydroxybenzoylester), 3beta,11alpha,15alpha-trihydroxylup-20 (29)-ene-11-(3′-methoxy-4′-hydroxybenzoyl)-15-(4′-hydroxy benzoyl ester), 3beta, 11alpha, 15alpha-trihydroxylup-20 (29)-ene-11,15-di(3′-methoxy-4′-hydroxybenzoyl ester), and 3beta, 11alpha, 15alpha-trihydroxylup-

20(29) -ene-11-(3′-methoxy-4′-hydroxybenzoyl)-15-(benzoyl ester). These compounds showed significant neuroprotective activities against glutamate-induced neurotoxicity in primary cultures of rat cortical cells[24].

To confirm the actions of such anti-inflammation and immunoregulation, the author investigated in this paper the effects and mechanisms of *Ulmus davidiana* Planch herbal extract solution(UDHES) on G-protein-AC-cAMP transmembrane signal transduction at levels of organ, cell, molecule, respectively. The results here showed that treatment with UDHES attenuated the progressive inflammatory-induced degeneration of synovia, cartilage, and bone in arthritic joints and suggested that it has important therapeutic potential in the treatment of autoimmune and inflammatory disorders and plays a relatively greater role in regulation of inflammatory arthritis in this model.

To clarify the mechanisms by which UDHES suppresses murine CIA, I studied the effects of UDHES on cellular and humoral immunity to CII in CIA rat. In this model, arthritis is characterized by paw edema, reduced body weight and the development of inflammatory lesions. The severity of inflammatory reactions in ankle joints of CIA rats, arthritis score and body weight gain was assessed, which were restored to normal by treatment of UDHES(Table 1, 2 and 3). These effects of UDHES in vivo on inflammation and bone destruction in arthritic rat result from direct inhibition of synoviocytes and osteoclast activity.

The synovial membrane connects the bones and lines the inner cavity of the joint together with the articular cartilage. The normal synovium is collapsed upon itself and onto the articular cartilage. The joint cavity contains a minimal amount of synovial fluid necessary for boundary-layer lubrication. The normal synovium consists of a lining that is two- to four-cell layers thick. RA is a synovial inflammatory disease marked by accumulation of T cells, plasma cells, macrophages, and other cells, increased numbers of blood vessels, and hyperplasia of the invasive intimal lining in which the synovial environment is characterized by intense immunological activity. The macrophage-like synoviocytes and fibroblast-like synoviocytes (FLSs) of the hyperplastic lining layer particularly exhibit an activated phenotype[25]. These cells are a major source of several pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, promoting induction of adhesion molecule and proteinase gene expression are found in synovium and play a major role in the progression of joint destruction[26]. These factors play an important role in attracting and activating other inflammatory cells and in the degradation of cartilage and bone. Both IL-1 and TNF-α are important in the pathogenesis of RA[27] and interactions between the two may produce synergistic effects. Contact between cells present in the rheumatoid synovium, notably T cells and macrophages, may be an important trigger for production of these cytokines. Some experimental evidence suggests that TNF-α may be more important in promoting mechanisms leading to inflammation, whereas IL-1 may be more important in processes leading to cartilage and bone destruction and in limiting mechanisms involved in cartilage repair. IL-1 is a polypeptide produced mainly by macrophages, which has multiple biological activities including induction of the production of acute phase proteins by hepatocytes, and stimulation of prostaglandins and collagenase production by synovial cells[28]. On the basis of these facts, IL-1 is regarded to be an essential mediator of inflammation. In particular, the importance of IL-1 in RA has been reported by various investigators[29]. For example, IL-1 production from RA synovium correlated not only with the degree of inflammation but also with that of joint destruction. Therefore, it is thought that an inhibitor of IL-1 generation could be a useful therapeutic agent in the treatment of RA. Agents that inhibit secretion of these cytokines, or that can block their binding to cell surface receptors, are increasingly being viewed as potential therapeutic agents that might provide

increased specificity compared with traditional drugs. Recent advances in anti-cytokine therapy confirm that these factors play a critical role in the pathogenesis of RA[30]. Development of novel treatment strategies that alter the cytokine milieu would be greatly facilitated by dissecting the regulatory elements that control mediator production in the joint.

In this study, the results indicated that UDHES could inhibit IL-1 and TNF-α level secreted by PM and synovial cells of the CIA model rats (Table 5, 6 and 7). It might be involved in its mechanisms of anti-inflammation and immunosuppression. The results presented here demonstrate a role for IL-2 during the early stages of CIA. IL-2 may be integral to many autoimmune processes. Further investigation of the mechanisms of action of IL-2 in autoimmunity may lead to a better understanding of the pathogenesis of autoimmune arthritis and other autoimmune disorders.

From other studies concerning pathogenesis of CIA in animals, it has been suggested that humoral immunity to type II collagen plays an important role. The anti-collagen titer in serum increased before onset of arthritis. The transfer of anti-collagen antibody obtained from arthritic mice can induce the arthritis. The acceleration of the severity of arthritis was attributed by treatments of IL-1, TNF-α, IL-6, IL-12, and IFN-α[29]. Thus, it seems that the suppression of cellular immune responses to CII could alter the sequential development of arthritis. The critical importance of antibody to type II collagen in the development of CIA is now clearly established. The importance of humoral immunity in CIA was indirectly emphasized by Schoen et al[31]. They questioned the role of cell-mediated immunity (CMI) to collagen in CIA. Recently, this laboratory provided direct evidences for an antibody-mediated pathogenesis of CIA by showing that IgG specific for type II collagen produces arthritis[32]. They showed that S.C. injection of RII-coupled spleen cells induced RII-specific CMI in rats but neither arthritis nor antibody to RII. These findings and those previously reported by others suggest that CMI to type II collagen alone is not sufficient to induce arthritis. However, these data do not exclude the importance of helper T cells in the genesis of immunity to type II collagen, a T-dependent antigen, nor the possibility that CMI may contribute to tissue injury once arthritis is initiated. CMI to CII is required in addition to humoral antibodies to CII for the sustained development of CIA. The clinical course of CIA is regulated by suppressor T cells and a reduction in the Lyt-2^+ cell ratio in the blood or regional lymph nodes has been reported during the development of murine CIA. UDHES significantly suppressed the delayed-type skin reaction to CII in CIA rat (Table 10). Its suppressive action on delayed-type hypersensitivity reactions takes place via the induction and/or activation of Lyt-2^+ cells. UDHES, therefore is believed to inhibit murine CIA through suppression of CMI responses to CII, possibly by induction and/or activation of suppressor T cells. UDHES had no effect on the level of serum anti-CII antibodies (Table 9). These results suggest that UDHES inhibits the development of CII-induced arthritis in rats by suppressing delayed-type hypersensitivity to CII.

Cyclic adenosine 3′, 5′-monophosphate (cAMP) is a classical second messenger and through changes in its rate of synthesis and degradation mediates the effect of a large number of hormones, autacoids and neurotransmitters thereby modulating processes as functionally diverse as visual phototransduction and anaesthesia. G proteins are biochemical transducers that operate as allosteric regulatory elements. The cAMP signaling pathway is an important mediator of extracellular signals in organisms from prokaryotes to higher eukaryotes. In mammals, two types of adenylyl cyclase synthesize cAMP; a ubiquitous family of transmembrane isoforms regulated by G proteins in response to extracellular signals. The changes of cAMP may reflect changes in gene expression and could result in changes in G-protein levels affecting signal transduction

pathways in chronically treated animals[13].

Cytokines probably play an important role in the development of CIA by a wide variety of signal generating. In CIA rats model, it has been shown that UDHES inhibits many cytokine-signal transduction, including IL-1, TNF-α and so on that were generated by G-protein-AC-cAMP pathway(Table 4-8). Therefore, the superior effect of UDHES on the CIA model may be due to the suppression mode of UDHES against cytokine signaling, which suggest that inflammatory reaction could be inhibited by UDHES. In previous mentioned GCS on CIA rats study[13], it showed the quite resembling effects on inflammatory and immune responses. The administration of GCS (30, 60, 120㎎/㎏ for 7days) reduced above changes significantly. GCS increased cAMP level of synoviocytes, which decreased in CIA rats in vitro. At the same time, GCS inhibited mRNA expression of Gi, and TNF-a of synoviocytes and increased mRNA expression of Gs of synoviocytes in CIA rats. GCS had no effect on the concentration of antibodies to CII. GCS possesses anti-inflammatory and immunoregulatory actions and has a therapeutic effect on CIA rats due to G protein- AC-cAMP transmembrane signal transduction of synoviocytes, which play a crucial role in pathogenesis of this disease.

Briefly, UDHES resulted in suppression of bone destruction and amelioration of inflammation in autoimmune rats through the intervention of intracellular signal transduction pathways in synoviocytes.

The results suggest that G-protein-AC-cAMP signal pathway plays critical roles in the inflammation and joint destruction in RA. It is that the signaling medicated by G protein coupled AC-cAMP may be one of the important mechanisms for the inflammatory -immunoregulation of UDHES[33,34].

The results presented in this study showed that UD warrants further investigation, including clinical study. UD, also has no adverse effects. Further studies to undertake UD herbal acupuncture are in progress.

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