Treatment with SI000413, a New Herbal Formula, Ameliorates Murine Collagen-Induced Arthritis

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Key words Pyrolae herba; Trachelospermi caulis; collagen induced arthritis; IL-6

Rheumatoid arthritis (RA) is one of the most common forms of systemic autoimmune disease that affects 0.5—1% of the adult population. RA patients present symptoms of acute polyarthritis with synovial hyperplasia and progressive cartilage and bone destruction, which results in chronic pain and functional disability. Current treatments include disease modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), steroid, and biological response modifiers such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) blocking agents, and other immuno-suppressive drugs.1,2) Therapies available to RA patients are targeted to slow down the progression of the disease and prevent further joint damage. This may lead patients to depend on long-term use of medication and often suffer from side effects. Therefore, much effort has been put into screening new therapeutic agents from natural products that might have anti-inflammatory activity and fewer adverse effects.

We created a new formula consisting of Pyrolae herba (PH) and Trachelospermi caulis (TC), designated as SI000413. From our screening, these two botanicals have anti-inflammatory activity and fewer adverse effects. Therefore, much effort has been put into screening new therapeutic agents from natural products that might have anti-inflammatory activity and fewer adverse effects.

We tested the effects of SI000413, a new formula, consisting of Pyrolae herba and Trachelospermi caulis, on type II collagen-induced arthritis (CIA). CIA was induced in DBA/1J mice by immunization with bovine type II collagen (CII) on days 1 and 21. SI000413 was orally administered 3 times per week throughout the experiment and indomethacin was served as a positive control. Clinical scores, the count of arthritic legs, levels of interleukin 6 (IL-6) and anti-CII antibody, and lymphocyte subsets in blood were examined. SI000413 suppressed CIA development in a dose dependent manner and reduced the incidence of arthritic legs in mice. Histological analysis showed administration of SI000413 reduced inflammatory signs and cartilage destruction. Serum levels of IL-6 and anti-CII antibody were significantly decreased in SI000413-treated mice and the percentages of CD4 T cell, CD8 T cell and B cell in blood were restored to normal levels. In conclusion, we demonstrate that SI000413 ameliorates CIA both clinically and histologically and inhibits the production of anti-CII antibody and pro-inflammatory cytokine in the CIA mouse. These findings suggest that SI000413 is a potential new therapeutic herbal formula for the treatment of RA.

MATERIALS AND METHODS

Animals Male DBA/1J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were kept in a temperature controlled room (at 23±1 °C) with a 12 h light/dark cycle, housed in polystyrene cages at Kyunghee University and given standard rodent chow and water ad libitum. Mice were cared for according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., U.S.A.). All experiments were approved by our institutional ethical committee for animal welfare. Mice were randomly separated into non-immunized (Normal), untreated immunized (Control), collagen-immunized SI000413 treated (SI000413), and collagen-immunized indomethacin (Sigma, St. Louis, MO, U.S.A.) treated (Ind) groups.

SI000413 Preparation SI000413 was prepared by mixing Pyrolae herba (PH) and Trachelospermi caulis (TC), obtained from Hunan Guohua Pharmaceutical Co., Ltd. in China, and authenticated by Dr. Yaojinpeng in Hunan Guohua Pharmaceutical Co., Ltd. The mixed material was chopped using a domestic mixer and 10 parts of 30% ethanol were added. After allowing the mixture to stand at 100 °C for 2 h, the ethanol extracts were filtered and concentrated in a 60—70 °C water bath under reduced pressure. The yield of the extract was approximately 2.5%. A voucher specimen (Sinil-000413) was deposited at Life Science R&D center, Sinil Pharmaceutical Co., Ltd. The extract power was stored...
at −20 ºC until use.

**Analysis of Arctiin in SI000413 by HPLC and UPLC**

For analysis of arctiin, diluted samples were filtrated through 0.2 µm (for UPLC) or 0.45 µm (for HPLC) microspin PVDF filter. The apparatuses were HPLC system equipped with a UV detector (Waters, Milford, MA, U.S.A.), or UPLC system (Waters, Milford, MA, U.S.A.). The constituents of the extract were separated by YMC-Pack Pro C18 RS column (5 µm, 4.6×250 mm, for HPLC system) or Waters ACQUTY UPLC \( ^{TM} \) BEH C18 column (1.7 µm, 2.1×100 mm, for UPLC system). The mobile phase consisted of 50% methanol, and the flow rate was 1.0 ml/min. The UV detector was set at an absorbance of 280 nm.

**Mouse CIA Model and SI000413 Treatment**

The CIA model was introduced as previously described.6) Bovine type II collagen (Chondrex, U.S.A.) was dissolved overnight at 4 ºC in 0.05 M acetic acid to 2 mg/ml. This solution was then emulsified in an equal volume of complete Freund’s adjuvant (CFA) (Chondrex, U.S.A.). On day 1, DBA/1J mice age 7 week were injected at the base of the tail with 0.1 ml of the emulsion. On day 21, a booster injection with the same amount was given. From the day of the first immunization until the end of the experiment, mice were administered with SI000413, indomethacin or vehicle only (water) three times a week, respectively. SI000413 was administered at a dose of 0.02, 0.2 or 0.4 g/kg and a dose of 1 mg/kg of indomethacin was determined as described previously.3)

**Evaluation of Arthritis**

Mice were monitored daily for signs of arthritis and each paw was scored individually as follows: 0 = normal, 1 = swelling of one digit, 2 = swelling of 2 or more digits, 3 = swelling of heel, 4 = joint deformity with ankylosis. Each mouse was assigned as arthritis score that equaled the sum of the scores for each paw, so that the possible maximum score per mouse was 16.

**Histopathologic Assessment**

Mice were killed and joints were removed, immediately fixed in 10% buffered formalin and decalcified in a decalcifying solution (9% formalin and 10% formic acid) for 1 week. The tissue was then processed and embedded in paraffin. Five-micrometer tissue section was prepared and stained with hematoxylin and eosin using standard methods.

**Determination of Anti-collagen Antibody in Serum**

Serum was collected at the end of the experiment. The level of anti-type II collagen (CII) antibody was measured using the ELISA method. Briefly, flat-bottom 96-well ELISA plates were coated overnight at 4 ºC with bovine CII. After blocking the plates with 10% fetal bovine serum-PBS, serum was diluted 1 : 20000, added to duplicate wells, and incubated for 1 h at room temperature. Plates were washed, and peroxidase-conjugated goat antibody to mouse IgG (Sigma, St. Louis, MO, U.S.A.) diluted at 1 : 100000 was added to each well and the plates were incubated at room temperature for 1 h. The plates were washed and then tetramethylbenzidine substrate solution (BD Pharmingen, San Diego, CA, U.S.A.) was added. After incubation at room temperature for 30 min, the stop solution (0.2 M \( H_2SO_4 \)) was added and the absorbance was measured at 450—570 nm. The data were expressed as the mean±S.D. of optical density values as described previously.6)

**Measurement of Circulating IL-6 in Serum**

Concentrations of IL-6 in serum were quantified using mouse IL-6 duoset ELISA kit (R&D Systems, U.S.A.). Each sample was assayed in duplicate and the concentrations were determined with the help of a standard curve.

**Flow Cytometric Analysis of Lymphocyte Subsets in Blood**

Blood samples were collected by cardiac puncture and red blood cells were removed by lysis. Cells were washed twice in PBS and resuspended to form a suspension of \( 1 \times 10^8 \) cells/ml. 0.1 ml of cell suspension was used for flow cytometric analysis to detect lymphocyte subsets by a double staining technique. To analyze B220+CD3− (B cell), CD3+CD4+ and CD3+CD8+ cell subsets, cells were stained with anti-CD3 FITC (CD3 e chain) (145-2C11), anti-CD8a FITC (Ly-2) (53-6.7), anti-CD4 P-E (L3T4) (GK1.5), and anti-CD45R/B220 (RA3-6B2) (antibodies for flow cytometry were purchased from BD Pharmingen, San Diego, CA, U.S.A.) for 20 min on ice in the dark. After washing twice with PBS/0.1% NaN3/1% FBS, the double-stained cells (live gated on the basis of forward and side scatter profiles) were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) and the data were processed using Cell Quest software (Becton Dickinson, San Jose, CA, U.S.A.).

**Statistical Analysis**

Data from the control or drug-treatment groups were analyzed using one-way analysis of variance followed by Dunnet’s post hoc test. Chi-square test was used to compare the count of arthritic legs in each group. The difference between the two means was assessed using non-paired Student’s t-test or Mann–Whitney \( U \) test (SPSS version 12). \( p \) values less than 0.05 were considered significant.

**RESULTS**

**HPLC and UPLC Analysis of Arctiin**

We chose arctiin as our reference material in the experiment because it has been shown to have an anti-inflammatory activity in RAW 264.7 cells.3) From the results of the standard calibration curve, \( R^2 \) value of arctiin was 0.9997 (data not shown), which exhibited a high linear relationship, and the content of arctiin in SI000413 was 0.8—1.6%. Figure 1 shows that arctiin was identified by matching the retention time against standard arctiin.

**Dose Dependent Effect of SI000413 on the Development of CIA**

Different doses of SI000413 and indomethacin were administered 3 times a week to DBA/1J mice from the day of the first CII injection until the end of the experiment for 6 weeks. The time course of clinical scores in each group is shown in Fig. 2A. We observed dose dependent reduction of the clinical scores by SI000413 treatment. Upon the termination of therapy, the clinical scores of the control mice reached 12.0±2.16. On the other hand, the scores were reduced to 9.89±5.95, 6.17±2.79, and 4.38±3.93 (\( p<0.05 \)) in the SI000413-treated groups (0.02 g/kg, 0.2 g/kg, 0.4 g/kg), respectively. Treatment with indomethacin recorded the lowest score, 3.78±3.10 (\( p<0.05 \)).

We also counted the number of arthritic legs in each group at all time points. Figure 2B shows that SI000413 treatment resulted in a significant reduction in the incidence of arthritic legs (\( p<0.001 \), by chi square test). The incidences of arthritis from the control reached from 69% at day 31 to 81% at
day 41, while those in SI000413-treated group were 34% (0.2 g/kg) ($p < 0.05$) and 16% (0.4 g/kg) ($p < 0.0005$) at day 31, and 45% (0.2 g/kg) ($p < 0.01$) and 36% (0.4 g/kg) ($p < 0.0005$) at day 41. The incidences in the indomethacin-treated group reached from 19% ($p < 0.0005$) to 41% ($p < 0.0005$) during the same period. On the whole, treatment with a dose of 0.4 g/kg and indomethacin showed comparable therapeutic effects on the progression of symptoms and incidence of arthritis in mice.

**Histological Findings** In order to confirm the inhibitory effects of SI000413 on our clinical assessments, we performed histopathology analysis. The control mice exhibited inflammatory cell infiltrate with extensive pannus formation, cartilage destruction and irregular joint space compared to the normal group (Figs. 3A, B). The SI000413 (0.2 g/kg) and indomethacin treated group showed limited synovial infiltration, well-kept articular cartilage and discernible joint space (Figs. 3C, D). Not only evaluations of clinical scores but also histological findings showed that the antiarthritic effect of indomethacin was more potent than that of SI000413.

**Effects of SI000413 on Serum Anti-CII IgG Antibody and IL-6 Levels in CIA Mice** CII-specific antibody is a major pathogenic factor in inducing arthritis following CII immunization. The level of anti-CII antibody was significantly lowered from 0.944 in the control mice to 0.754 ($p < 0.05$) and 0.755 ($p < 0.05$) in the SI000413 (0.2 g/kg) and indomethacin treated mice, respectively (Fig. 4). Given the importance of anti-CII antibody, our data suggest that a reduction in anti-CII antibody is associated with the suppressive effect of SI000413 on CIA.

Next we examined the effect of SI000413 on serum IL-6 level. It was reported that one of the earliest indicators of the systemic response in RA is an elevation in serum IL-6 levels. As shown in Fig. 5, SI000413 treatment signifi-
Significantly reduced the level from 230.5 pg/ml in the control mice to 34.2 pg/ml ($p<0.005$) by 86% in the SI000413-treated mice. However, despite its remarkable effect on arthritis, indomethacin slightly reduced IL-6, which confirmed previous research in which some NSAIDs, including indomethacin, did not affect IL-6 secretion.12)

**Lymphocyte Subpopulation Change in Blood** Because the pathogenesis of CIA and RA involves both T cell and B cell immunity to CII, we conducted flow cytometric analysis of lymphocyte subsets in blood from each group. The control mice showed reduced levels in all lymphocyte subsets compared to the normal mice. It should be pointed out here that, in our flow cytometric analysis, a lower percentage of B cells in the control mice was observed than that of the normal mice, although the absolute count of B cells would be much higher in the control mice. This is probably due to the fact that when we performed cell surface staining by flow cytometry, we did not separate lymphocytes from whole blood cells and it is possible that other types of blood cells were contaminated within whole blood samples. All lymphocyte subsets in SI000413 and indomethacin treated groups were increased compared with the control group (Fig. 6). In particular, a significant increase in the percentage of B cells was observed in the SI000413-treated mice, which did not occur in indomethacin treatment. Considering the same levels of anti-CII antibody that each group produced, their underlying mechanisms in diminishing the antibody response to CII seem to be quite different.

**DISCUSSION**

The present study examined the therapeutic application of
a new herbal formula, SI000413 in the mouse model of CIA, which is widely used to study rheumatoid arthritis (RA). We demonstrated that treatment with SI000413 significantly reduced clinical symptoms in DBA/1J mice immunized with CII. SI000413 also significantly decreased the formation of anti-CII antibody while maintaining higher percentages of T and B cells in whole blood from CIA mice. In addition, the level of serum IL-6 was remarkably lower in the SI000413 treated mice.

The etiology of RA is still unknown but several foreign antigens and autoantigens have been postulated to contribute to the development of RA, generating clones of autoreactive B cells.13—16 The CIA experimental model uses heterologous CII to develop arthritis since it is easier to induce arthritis than autologous CII.17 Therefore, it can be stated that the CIA model is achieved by the T cell or B cell response to foreign antigen, which can directly bind to the joint cartilage of mice.5,18

The current standard of care for RA is a combination of DMARDs, for example, methotrexate-sulfasalazine therapy or methotrexate-doxycline therapy. However, the simultaneous use of combination of DMARDs is controversial because it can increase the risk of toxicity as well as efficacy.19,20 To reduce the risk of adverse events and the frequent use of steroid, the combination of herbal therapeutic agents may be useful to slow down the progression of the disease and further joint damage.

As previously described, the components of SI000413, Pyrolae herba and Trachelospermid caulis, had anti-inflammatory activity by downregulating the expression of iNOS and TNF-α in Raw264.7 cells.3,4 Although we did not directly measure the local secretions of pro-inflammatory cytokines or mediators in the joint synovium, our clinical assessments and histological findings clearly showed that SI000413 reduced joint inflammation. Impressively, SI000413 decreased the serum concentration of IL-6 by 86% compared with the control group. IL-6, secreted mostly by macrophages and endothelial cells, is known to be involved in the development and clinical manifestations in RA, and absence of IL-6 signal transduction is reported to reduce the development of CIA in mouse.11 Although it would be premature to conclude that systemic reduction of IL-6 by SI000413 directly indicates a decrease in local inflammation, it seems that SI000413 exerts anti-inflammatory effect on systemic macrophages as well as local inflammatory cells. Further studies are needed to clarify the local effect of SI000413 on IL-6 secretion in the joint.

Anti-CII antibody production contributes to the development of CIA evidenced by reports that the passive transfer of anti-CII sera elicits arthritis.9,10,21,22 The role of anti-CII antibody in CIA is to activate the complement system, recognize the surface of cartilage and initiate the inflammatory responses.23 In keeping up with the reduced level of IL-6, a decrease in serum anti-CII level may help to explain how SI000413 can ameliorate arthritic symptoms in DBA/1J mice. This also brings us to speculate on the possible role of SI000413 in stabilizing the recognition of CII as harmful antigen because it seems to convey some inhibitory signals in evoking innate and specific immunity.

Since chronic inflammatory disease most often involves inappropriate immune responses, many of anti-inflammatory medications are inevitably aimed at blocking interactions between various immune cells and pro-inflammatory cytokines. This accounts for the fact that the long term use of DMARDs or any drugs that modifies the immune responses can cause infection, which is an indicator of a weakened immune response in RA patients.13 Therefore, we evaluated whether SI000413 treatment affected the percentage of lymphocyte subsets in whole blood using flow cytometry. Our results showed that SI000413 restored the proportions of CD4+ and CD8+ T cells and B cells to normal levels. Interestingly, indomethacin significantly elevated the percentages of CD4+ and CD8+ T cells but the effect on B cells was minimal compared with SI000413. As mentioned previously, it is not clear how SI000413 can interfere with the generation of anti-CII antibody, although both SI000413 and indomethacin reduced the production of anti-CII antibody to a similar degree. We suspect that SI000413 may play some inhibitory role in recognition of CII as immunogen, thus exerting anti-CIA effects. Further investigations of in vitro response of T cells or B cells need to be performed to demonstrate the protective mechanism of SI000413 in preventing a hyper-response of CII-specific T cells or B cells.

Arctii, a glycoside of arctigenin, is abundant in TC, and we have previously confirmed arctii to play some inhibitory role in LPS-induced NO production.3 From our HPLC and UPLC data, we found that the content of arctii is very high in SI000413 extract, which may contribute to the anti-inflammatory activity of SI000413.

Taken together, our data presented that SI000413 reduced arthritic symptoms in CIA mice by inhibiting the production of IL-6 and anti-CII antibody but without much disturbance in lymphocyte populations. This provides pharmacological basis for the use of SI000413 in potentially efficacious and safe RA therapy.

Acknowledgments This work was supported by Sinil Pharmaceutical Co., Ltd.

REFERENCES