

## The Population of CD40L-expressing Cells was Slightly but not Significant Decreased in Lymphoid Tissues of Collagen Induced Arthritic Mice Treated with Hochu-Ekki-To

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**Objective:** To clarify the mechanism of the action of Hochu-Ekki-To (HET) on collagen-induced arthritic (CIA) mice by analyzing the CD40L-expressing cells population. **Methods:** CIA was induced in male DBA/1J mice by immunization with two injections of bovine type II collagen (CII). HET or water was orally administered. The subpopulations of lymphocytes obtained from lymph nodes and spleen were detected at 3 weeks after boost using flow cytometry. **Results:** Although the population of CD4<sup>+</sup>CD40L<sup>+</sup> cells tended to be decreased in the HET group compared to that in control mice, there was no significant difference between the two groups. These findings were observed in lymphocytes obtained from both lymph nodes and spleen. **Conclusion:** HET suppresses the development of CIA. These effects may be partially induced via the decrease in the population of CD4<sup>+</sup>CD40L<sup>+</sup> cells, but the role of this action is probably limited.

**Key words**—Hochu-Ekki-To; collagen-induced arthritis (CIA); immunomodulation; alternative medicine; CD40L

### INTRODUCTION

Type II collagen (CII)-induced arthritis (CIA) is a useful experimental model for studying the pathological mechanisms of therapeutic agents for human rheumatoid arthritis (RA). The development of CIA is known to depend on the presence of activated CD4<sup>+</sup> T cells and the disease is associated with both cell-mediated and humoral immunity to collagen.<sup>1,2</sup> Especially, the importance of CD40-CD40L ligation in the development of CIA has been illustrated.<sup>3</sup>

Hochu-Ekki-To (HET), a herbal formula, is composed of ten species of medicinal plants and used for chronic diseases or weakness after illness.<sup>4</sup> HET has been widely used to treat patients with certain immune-related diseases. Several studies have shown that HET formula also exhibits immunopharmacological activities by regulating certain cytokines.<sup>5–8</sup> Our previous report showed that HET caused a reduction of soluble CD23, a marker of activated B cells in a patient with RA, as well as improvement in

joint symptoms.<sup>9</sup> Furthermore, we previously demonstrated using a CIA mouse model that suppression of the secretion of proinflammatory cytokines TNF- $\alpha$  and IL-6, and inhibition of B cell activation participate in the mechanism of the anti-arthritis activity of HET.<sup>10</sup> However, we have not completely clarified the mechanism of this action.

This study focused on the CD40L molecule, and investigated the population of CD40L-expressing cells on lymphocytes in a CIA model treated with HET.

### MATERIALS AND METHODS

The animal experiment committee in Toyama University approved our experimental protocol, and the animal experiments were performed according to the guidelines of the Japan Society for the Promotion of Science.

**Animals** Eight-week-old male DBA/1J mice purchased from Sankyo Laboratories, Japan, were used. The mice were kept in a temperature-controlled room with a 12-h light/dark cycle, housed in polystyrene cages and given standard rodent chow and water *ad libitum*. Mice were randomly assigned to untreated

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CII-immunized (CONT) and CII-immunized HET-treated (HET) groups.

**Hochu-Ekki-To (HET) Preparation** HET was prepared by mixing ten herbals components (purchased from Tochimoto Co., Ltd. Osaka, Japan), all in dried crude form, and preparing an extract from the mixture as previously reported.<sup>10</sup> In brief, 120 grams of mixed material was added to 500 ml of distilled water and heated for 60 min at 100°C. The extract was filtered, and the residue was extracted a second time by the same procedure. The insoluble substances were removed by centrifugation and the supernatant was concentrated by rotary evaporation, and lyophilized to form a dried powder.

#### Introduction of Collagen-induced-arthritis and Hochu-Ekki-To Treatment, and Clinical Assessment

The immunization reagent was freshly prepared as follows: Bovine type II collagen (K42) (Sankyo Laboratories, Japan) was dissolved in 0.1 M acetic acid at 2.0 mg/ml. Then the solution was emulsified in an equal volume of complete Freund's adjuvant (CFA, DIFCO Laboratories, Detroit, Michigan, USA). CIA was induced in DBA/1J mice by intradermal injection of 0.2 ml of emulsion into the base of the tail. Three weeks later, a second immunization was carried out in the same manner. The day of the second immunization was considered day 0. From the day of the first immunization until the end of the experiment, HET-treated and control mice were administered HET or vehicle only (water) daily using a gastro-tube. HET was administered at 0.5 grams powder/kg/day. Arthritis was assessed clinically using the arthritis score.<sup>10,11</sup>

#### Flow Cytometric Analysis of Lymph Cell Subsets in the Lymph Nodes and Spleen

Mice were sacrificed on day 21 after the second immunization based on the findings of our previous study.<sup>10</sup> The lymphoid tissues (axillary and inguinal lymph nodes, and spleen) were dispersed in PBS using a fine stainless steel mesh. The lymphocyte suspension was collected and treated for 5 min in hemolytic buffer (NaCl 138 mM, KHCO<sub>3</sub> 0.01 M, 2NaEDTA 0.1 mM, pH 7.4) to remove erythrocytes, and the lymphocytes were washed twice in PBS and collected by centrifugation at 1500 rpm for 15 min. Then the cells were resuspended in PBS at a concentration of 1 × 10<sup>6</sup> cells/ml. One ml of cell suspension at 1 × 10<sup>6</sup> cells/ml was used for flow cytometric analysis to detect lymphocyte subsets by a double staining technique. To ana-

lyze CD40L<sup>+</sup> and CD4<sup>+</sup>CD40L<sup>+</sup> lymphocyte subsets, 1 × 10<sup>6</sup> cells were stained by incubation with 10 μl of fluorescein isothiocyanate (FITC)-labeled anti-mouse CD40L monoclonal antibodies (mAb) and 10 μl of phycoerythrin (PE)-labeled anti-mouse CD4 antibody (Immunotech, Marseille, France) respectively, at room temperature for 45 min. After incubation, the suspension was analyzed using a flow cytometer (Epics XL, Beckman Coulter, France).

**Statistical Analysis** The data were analyzed by repeated measures ANOVA, or Mann Whitney U test using Staviw v 4.5 software. *p* < 0.05 was considered significant.

## RESULTS

#### Suppressive Effect of HET on the Development of CIA

Neither the CONT nor HET groups showed any symptoms before the second CII injection. We confirmed the suppression of the clinical progression of CIA by HET treatment (Fig. 1). In this experiment, HET treatment was performed at 0.5 g/kg, because there was no significant difference between 0.5 and 2.5 g/kg of HET as previously indicated. In the CONT group, the arthritis severity was significantly more serious than that in the HET-treated group at all indicated time points. There were fewer mice with swelling of the entire paw in the HET-treated groups.

Concerning the incidence of CIA development in CII-immunized mice, HET treatment resulted in a significant reduction in the incidence of CIA similar to the reduction in the previous experiments.<sup>10</sup>

#### CD40L<sup>+</sup> Subset Partition Change in Lymphoid

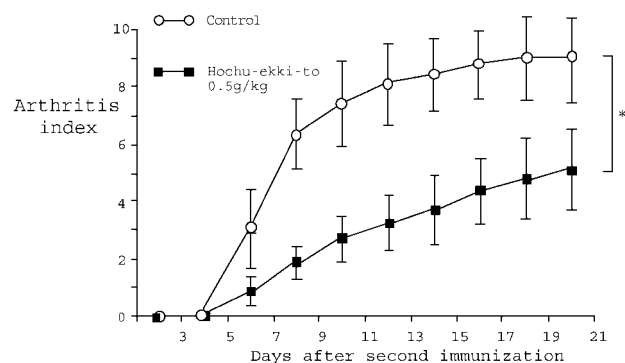


Fig. 1. Suppressive Effect of HET Treatment on the Progression of CIA

CIA was induced in DBA/1J mice by two injections of CII. Mice were orally treated from the day of the first injection with 0.5 g/kg (HET 0.5: n=15) or untreated (CONT: n=15). The arthritis index in each group is presented as the mean ± S.E. \**p* < 0.001 compared with the control group by repeated measures ANOVA.

Table 1. Lymphocyte Subset Fractions in Lymph Nodes and Spleen

Lymphoid tissues	Lymph nodes		Spleen	
	CD40L <sup>+</sup>	CD4 <sup>+</sup> CD40L <sup>+</sup>	CD40L <sup>+</sup>	CD4 <sup>+</sup> CD40L <sup>+</sup>
HET mice (N=15)	2.9±2.0 <sup>a)</sup>	1.2±1.1	2.8±1.4	1.2±1.6
CONT mice (N=15)	3.2±1.2	1.5±0.9	3.0±1.4	1.5±1.0

<sup>a)</sup> percentage mean±SD. There was not significant difference between HET mice and CONT in the percentage of fractions, although fewer activated T cells expressed CD40L in HET mice than in CONT.

**Tissues** Table 1 shows the CD40L<sup>+</sup> subset partitions in lymph nodes and spleen derived from CONT and HET mice, respectively. The population of total CD40L<sup>+</sup> cells was lower in HET than in CONT, and the population of CD4<sup>+</sup>CD40L<sup>+</sup> lymphocytes obtained from lymph nodes was also lower in HET than in CONT, but the differences were not significant. We further analyzed the CD40L<sup>+</sup> cell fraction in lymphocytes obtained from the spleen. Although the populations of both total CD40L<sup>+</sup> cells and CD4<sup>+</sup>CD40L<sup>+</sup> cells were decreased in splenic cells from HET compared to those from CONT mice, the differences were not significant. Concerning the CD40 molecule on B cells, we confirmed that there were no changes in the population of B220<sup>+</sup>CD40<sup>+</sup> B cells between HET and CONT mice.

## DISCUSSION

Traditionally, a water decoction of HET is commonly used in oriental medicine to treat patients with chronic diseases including RA or weakness after illness.<sup>4)</sup> We previously reported the successful use of HET to treat a patient with RA<sup>9)</sup> and demonstrated that HET exerts anti-CIA effects via anti-inflammatory and immunomodulatory activities.<sup>10)</sup> However, we have not completely clarified the mechanism of this action, although it has been demonstrated that suppression of the secretion of the proinflammatory cytokines TNF- $\alpha$  and IL-6, as well as inhibition of B cell activation participate in the mechanism of the anti-arthritis activity of HET. In this study, furthermore, we demonstrated the action of HET in CIA mice by investigating the population of CD40L-expressing cells.

CD40L is transiently expressed on activated T cells, mainly the CD4<sup>+</sup> subset. The importance of CD40-

CD40L ligation in the development of autoimmune disease has been illustrated in several murine models of autoimmunity by applying blocking antibodies.<sup>3,12)</sup> In CIA, blocking B cell activation by treatment with anti-CD40 ligand leads to protection against the disease and a total block of the antibody response.<sup>3)</sup> Other investigators demonstrated that the administration of stimulatory anti-CD40 mAb resulted in earlier onset and more severe disease using CIA mice.<sup>13)</sup> These observations suggest that the level of CD40 activation during the induction of an autoimmune response may determine the severity of the resulting disease. In our experiments, the populations of both CD40L<sup>+</sup> cells and CD4<sup>+</sup>CD40L<sup>+</sup> T cells in the lymphocytes obtained from lymph nodes and spleen tended to be decreased in HET mice compared to that in CONT mice, although the difference was not significant. We speculate that these effects, the suppression of T cell activation, may partially contribute to the improvement of joint damage. However, we did not consider suppression of T cell activation by HET to be the main mechanism in the relief of arthritis, since inhibition of B cell activation such as suppression of anti-CII antibody production and IL-6 secretion<sup>10)</sup> participates in the mechanism of the anti-arthritis activity. It is possible that HET inhibits the activation of B cells after acceptance of the signals through CD40 from CD40L on activated T cells. It has recently been demonstrated that anti-CD80/86 treatment inhibited the disease score and incidence, whereas anti-CD28 treatment led only to delayed disease onset in CIA mice.<sup>11)</sup> These phenomena suggest that the cross-talk between activated B cells and activated T cells by CD40-CD40L ligation may be more crucial than that between antigen-presenting cells and T cells upon recognition of antigen: CII through CD28 to CD80/86 molecule in the development but not onset of CIA. Therefore, several further experiments such as the investigations of CD80/86 molecule on B cells should be required to clarify our hypothesis that HET inhibits the activation of B cells.

Finally, it was confirmed that HET suppresses the development of CIA. These effects may be partially induced *via* the inhibition of the activation of T cells as a result of the decrease in the population of CD4<sup>+</sup>CD40L<sup>+</sup> cells, but the role of this action is probably limited.

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