The Effect of Keishi-bushi-to on Collagen-Induced Arthritis

Keizo WAKABAYASHI, Makoto INOUE, and Yukio OGIHARA*

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan. Received October 24, 1996; accepted December 25, 1996

To evaluate the usefulness of a traditional Chinese medicine (Kampo prescription), Keishi-bushi-to (KBT), which is composed of five medicinal plants derived from Kampo prescriptions used to treat rheumatoid arthritis, we investigated the effect of KBT on the development of arthritis induced by type II collagen (CII). Oral administration of KBT at a dose of 500 mg/kg from 7 d before intradermal injection of CII significantly reduced the severity from 7 d after the onset of arthritis. The reduction in body weight resulting from the development of arthritis was not seen in rats treated with KBT.

Plasma IgG and IgM anti-CII antibody levels were lower in KBT-treated rats than control rats. In addition, the clearance of IgG anti-CII antibody from circulating blood after intravenous injection was faster in KBT-treated rats than control rats. These results indicate that KBT is effective in suppressing collagen-induced arthritis and its effect is at least partly due to the suppression of humoral and cellular immunity.

Key words Keishi-bushi-to; traditional Chinese medicine; collagen-induced arthritis; rheumatoid arthritis; immune response

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that is associated with several immunologic abnormalities. To study the basis of the development of RA or to exploit therapeutic agents for RA, many animal models have been used such as adjuvant-, proteoglycan-, Staphylococcus aureus- and methylated bovine serum albumin-induced arthritis in addition to collagen-induced arthritis (CIA). Among such models, CIA, which was developed by Trentham, has been widely used as an animal model of autoimmune polyarthritis in mice, rats and primates, because antibodies to type II collagen (CII) have been detected in the serum and synovial fluid of some RA patients and, clinically and histologically, it resembles rheumatoid arthritis suggesting that autoimmunity to collagen may contribute to the disease process. The development of CIA is known to be related to both cellular and humoral immune responses to CII because passive transfer of T lymphocytes sensitized with CII or immunoglobulin G specific for CII induces arthritis. In addition, a number of cytokines, such as interleukin 1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α), are known to be involved in the arthritic process. Actually, antibodies to IL-1 and TNF-α prevent the onset of the disease and IL-10 and IL-4, which suppress IL-1, IL-6 and TNF-α production, ameliorate its progression. Furthermore, interferon-γ and transforming growth factor β have a protective effect against CIA without exerting a major effect on the production of anti-CII antibody. In a recent study, oral administration of CIA to patients with rheumatoid arthritis showed a significant improvement in the progression and its effectiveness was also verified in mice and rat CIA models. The enthusiastic exploitation of therapeutic agents for rheumatoid arthritis has shown that immunosuppressive drugs, cyclophosphamide, FK506, a microtubule stabilizer, taxol, an angiogenesis inhibitor, AGM-1470, anti-Fas antibody in addition to anti-rheumatic and anti-inflammatory drugs are effective in suppressing CIA, although their clinical application to rheumatoid patients requires much more investigation. Recent studies have provided a deeper pathological and immunological understanding of the development of arthritis, but the exploitation of antirheumatic agents without severe side-effects is still at an early stage. In the treatment for rheumatoid arthritis, some Japanese-Chinese traditional herbal medicines (Kampo prescriptions), were found to be effective clinically and their application depended on the severity or degree of development of the arthritis. Kampo prescriptions usually consist of several medicinal plants and are thought to be adapted for chronic diseases. Polysaccharides, universally present in medicinal plants, are well known to have immuno-modulatory activity and glycyrrhizin, saikosaponin and bicalin are also known to exhibit anti-inflammatory or anti-allergic actions. We have so far studied their pharmacological and biochemical actions in several diseases. Therefore, in this study we selected Keishi-bushi-to (KBT), a constituent of medicinal plants used in Kampo prescriptions and frequently applied in the treatment of rheumatoid arthritis and examined its pharmacological and biochemical effects on CIA using the Lewis rat CIA model to identify a scientific basis for its usefulness.

MATERIALS AND METHODS

Materials Soluble-form type II collagen (K42) was obtained from Collagen Gijutsukenshukai (Tokyo, Japan). Incomplete Freund’s adjuvant was from Difco Laboratories (Detroit, MI, U.S.A.).

Animals Female Lewis inbred rats at 8 weeks of age were purchased from Charles River Laboratories (Tokyo, Japan). They were housed in a temperature-controlled room (at 23 ± 1°C) with lighting from 7 a.m. to 7 p.m. and received standard laboratory chow (CE-2, Nippon Crea Co., Ltd., Shizuoka, Japan) and drinking water ad libitum.

KBT KBT (spray-dried aqueous extract) was obtained from Tsumura Co., Ltd. (Tokyo, Japan). KBT (human dose/d) consists of Cinnamomi Cortex 4 g, Zingibiris
Rhizoma 3 g, Glycyrrhizae Radix 2 g, Zizyphi Fructus 3 g and Aconiti Tuber 1 g. KBT was orally administered at 500 mg/kg/d, a dose ten times higher than the human daily dose, by syringe filled with 18G ball-point needle.

**Induction of CIA** Female Lewis rats were immunized with 0.5 mg bovine type II collagen in 0.5 ml 0.1 M acetic acid emulsified with an equal volume of incomplete Freund's adjuvant by intradermal injection at the base of the tail. Arthritis severity was determined using a grading system for each paw as follows: 0 = no arthritis; 1 = redness or swelling in paws or toes; 2 = severe swelling and/or joint deformity; and 3 = joint ankylosis. The score of each limb was summed, thus giving a severity range from 0 to 12 for each animal. The mean arthritis scores represent the severity only in arthritic animals per group.

**Anti-CII Antibody Quantification** An enzyme-linked immunosorbent assay (ELISA) was used to quantitate antibody to CII. ELISA multiplates (H type, Sumitomo-bakelite Co., Ltd., Tokyo, Japan) were coated with soluble bovine CII at a concentration of 5–20 μg/ml in phosphate-buffered saline (PBS) by incubating at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20 (PBS–Tween) and incubated with 1% bovine serum albumin-containing PBS for 30 min at room temperature. Plates were thoroughly washed three times with PBS–TWEEN 20 and then incubated with diluted serum 1:50–500 in PBS for 1 h at room temperature. After washing with PBS–TWEEN, peroxidase-conjugated rabbit anti-rat IgG or IgM antibody (Zymed Laboratories Inc., CA, U.S.A.) diluted 1:4000 in PBS was added to each well. 1 h later, plates were washed with PBS–TWEEN thoroughly and then developed with orthophenylenediamine (OPD) as the substrate. 40 μl OPD was dissolved in 100 ml citrate-phosphate buffer, pH 5.0, to which 40 μl 30% H₂O₂ was added immediately before use. The absorbance was determined by using a microplate reader (Model 450, Bio Rad, CA, U.S.A.) at a wavelength of 490 nm and a reference wavelength of 405 nm.

**Preparation of Serum Concentrate to Study Antibody Clearance** Immune sera were obtained from Lewis rats 4 weeks after immunization with CII. The sera were pooled and an equal volume of saturated solution of ammonium sulfate was added to precipitate immunoglobulin. The precipitate was collected by centrifugation at 15000 × g for 30 min, redissolved in a minimal volume of water and dialyzed against saline. The volume was then adjusted to 50% that of the original serum, and the concentrate was sterilized by filtration through a 0.22 μm microporous filter. The concentrate was administered immediately to rats intravenously via a tail vein.

**Statistics** Data were represented as means ± S.E. with the numbers of animals in parentheses. Statistical significance was determined by Student's t-test or the Wilcoxon test. Arthritis incidence between groups was analyzed using the χ² test. p values less than 0.05 were considered significant.

**RESULTS**

Lewis rats are known to develop arthritis after a single intradermal injection of native CII. 7 We therefore gave 500 mg/kg of KBT orally to Lewis rats from 7 d before the immunization with CII and water, instead of KBT, to control rats. The incidence of arthritis was similar in the KBT-treated (85%) and control groups (90%), whereas the onset in the KBT-treated group was significantly prolonged compared with the control group (12.9 d vs. 11.4 d, p < 0.01 by Wilcoxon test) (Fig. 1). When the severity of arthritis was measured for the entire period of the experiment, KBT treatment caused a significant

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**Fig. 1.** Effect of KBT on the Incidence of CIA in Lewis Rats

KBT, 500 mg/kg/d, was orally administered to female Lewis rats from 7 d before CII injection i.d. to the end of the experiment. Control rats were given water instead of KBT. ○, control group (n = 15); ●, KBT group (n = 15).

**Fig. 2.** Effect of KBT Administration Prior to Immunization on the Severity of Arthritis.

KBT, 500 mg/kg/d, was orally administered to Lewis rats from 7 d before CII injection i.d. to the end of the experiment. The severity of arthritis was represented as a mean arthritis score and rats without clinical signs of arthritis were precluded from the statistical analysis. (A) Arthritis scores on the day after immunization with CII were analyzed. (B) Arthritis scores on the day after the onset of arthritis in each rat were analyzed. ○, control group (n = 15); ●, KBT group (n = 15). **p < 0.05; ***p < 0.01 vs. control group, by Student's t-test.
suppression of the severity of arthritis from day 13 after immunization (Fig. 2A). When the day of onset was adjusted to day 1 for analysis, the development of arthritis in an early phase was clearly suppressed, resulting in amelioration of the subsequent arthritis (Fig. 2B). Figure 3 shows the change in body weight during the course of the experiment. Control rats exhibited a reduction in body weight after the onset of arthritis and the average body weight was about 10 g lighter in control group than the KBT-treated group. When KBT was orally administered from the day of immunization, the severity of the arthritis was slightly, but significantly, suppressed from day 27 (Fig. 4A). Furthermore, when the day of onset was adjusted as shown in Fig. 4B, the progression of arthritis in the early phase 7d from the onset was slower in the KBT group compared with the control group, resulting in a moderate suppression of arthritis. In general, the collagen-induced arthritic rat is known to have high plasma anti-CII antibody levels, although the reverse is not necessarily true. Therefore, serum IgG and IgM anti-CII antibody levels were determined every week by an ELISA assay as shown in Fig. 5. Both IgG and IgM anti-CII antibodies were reduced for the entire period in the KBT-treated group, suggesting that their production was suppressed or their elimination enhanced. We next determined the clearance of IgG anti-CII antibody after intravenous injection. The antibody concentrate was prepared from the serum of collagen-induced arthritic rats by ammonium sulfate precipitation. Rats treated with KBT, 500 mg/kg for 1 week, were injected with serum concentrate and then the concentration of IgG anti-CII antibody was determined 3, 6, 12, 24, 48 and 96 h later by ELIZA assay (Fig. 6). The early clearance rate was faster in the KBT group than in the control group and there was no difference in the serum level in both groups 96 h after injection.

Fig. 3. Change in Body Weight during the Course of CIA Development
Body weight of rats in the control (○) and KBT (●) groups was measured every 3d. KBT, 500 mg/kg/d, was orally administered to Lewis rats from 7d before CII injection i.d. to the end of the experiment. *p<0.05; **p<0.01 vs. control group, by Student's t-test.

Fig. 4. Effect of KBT Administration after Immunization on the Severity of Arthritis
KBT, 500 mg/kg/d, was orally administered to Lewis rats from the day of CII injection i.d. to the end of the experiment. The severity of arthritis was represented as a mean arthritis score and rats without clinical signs of arthritis were precluded from the statistical analysis. (A) Arthritis scores on the day after immunization with CII were analyzed. (B) Arthritis scores on the day after the onset of arthritis in each rat were analyzed. ○, control group (n=15); ●, KBT group (n=15). *p<0.05; **p<0.01 vs. control group, by Student's t-test.

Fig. 5. (A) IgG and (B) IgM Anti-CII Antibody Levels during the Course of CIA Development
Anti-CII antibodies were determined by ELISA assay every 7d as described in the Materials and Methods. KBT, 500 mg/kg/d, was orally administered to Lewis rats from 7d before CII injection i.d. to the end of the experiment. ○, control group (n=15); ●, KBT group (n=15). *p<0.05; **p<0.01 vs. control group, by Student's t-test.
DISCUSSION

In this study, oral administration of KBT was found to be effective in preventing the development of collagen-induced arthritis. Although no difference in the incidence of arthritis was observed between KBT and control groups, the onset of arthritis in KBT-treated rats was slightly prolonged, suggesting that the effect of KBT is moderate, but significant. Its efficacy was more potent when KBT was administered from 7 days before immunization with CII than when administered from the day of immunization, indicating that administration for a long period efficiently modulates the immune response to CII, resulting in suppression of the development of arthritis. IgG and IgM anti-CII antibodies in plasma were reduced in the KBT group throughout the study, indicating that the production or clearance of anti-CII antibodies was suppressed. Actually, when the clearance of IgG anti-CII antibodies was measured after intravenous injection of serum concentrate prepared from arthritic rats, KBT treatment stimulated their elimination. Although a high level of anti-CII antibodies is not closely linked to the development and severity of CIA, the production of anti-CII antibodies induces arthritis.\(^\text{11)}\)

Therefore, the action of KBT in reducing plasma anti-CII antibody levels seems likely to contribute to the suppression of CIA. However, administration of KBT after CIA injection showed a slightly weaker effect, indicating that the action of KBT may result from an immunosuppressive effect on the early phase of arthritis and an anti-inflammatory effect following the onset of arthritis. Autoantibodies such as anti-DNA antibody and anti-collagen antibody are produced by polyclonal activation of B lymphocytes in autoimmune diseases,\(^\text{28)}\) and their circulating concentration increases with age.\(^\text{29)}\)

However, in the CIA model, T lymphocyte activation is intimately related to nonspecific B lymphocyte activation, resulting in the development of arthritis, whereas the depression of T lymphocyte function may contribute to the nonspecific B lymphocyte activation and the decline in immunity accompanying by senility. Thus, the mechanism of polyclonal B lymphocyte activation is complicated, but KBT seems likely to modulate the enhanced immunity elicited by CIA challenge to suppress CIA. In conclusion, KBT, a Kampo-prescription, delayed the onset of CIA and reduced its severity. The mechanism by which this Kampo-prescription exhibited anti-CIA action was likely due to the suppression of immunity, as shown by the reduced anti-CII antibody and the acceleration of antibody clearance from circulating blood. We believe that this study provides a scientific basis for the use of Kampo medicines in rheumatoid arthritis.

Acknowledgements We thank Miss Nobuyo Kawai and Shoko Hayashi for their technical assistance.

REFERENCES


