Suppressive Effect of Dai-bofu-to on Collagen-Induced Arthritis

Makoto Inoue,* Yuka Ono, and Hajime Mizukami

Laboratory of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan. Received January 14, 2004; accepted March 2, 2004

Dai-bofu-to (DBT) is a traditional Japanese herbal medicine (Kampo medicine) used for the treatment of rheumatoid arthritis (RA). In the present study, to establish the usefulness of DBT, we examined the effect of DBT on collagen-induced arthritis (CIA). DBT (1.72 g/kg/d) significantly reduced the severity of arthritis throughout the experiment and significantly delayed the onset of arthritis. The induction of CIA decreased T cells and increased B cells in popliteal lymph nodes close to the affected joints, while the treatment of CIA with DBT counteracted the changes in T and B cells. In pX transgenic mice as a spontaneously developed arthritis model, a decrease in T cells and increase in B cells in popliteal lymph nodes were observed, as compared to BALB/c mice, the littermates of pX transgenic mice. In contrast, DBT returned the cell number of T and B cells to the level of BALB/c mice. As osteoclastogenesis is regulated by some T cell cytokines and osteotropic factors, we examined the effect of DBT on the receptor activator of NF-κB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG) and M-CSF mRNAs, which were induced by arthritis induction. Although DBT had no effect on RNK or RANKL mRNA levels, DBT stimulated an increase in OPG mRNA levels and suppressed an increase in M-CSF mRNA level. These results suggest that DBT may possess an anti-osteoclastogenetic effect, which is brought by reducing the ratio of RANKL/OPG and by decreasing M-CSF mRNA levels. In conclusion, immunomodulatory and anti-osteoclastogenetic effects might be involved in the suppression of arthritis by DBT.

Key words Dai-bofu-to; collagen-induced arthritis; bone destruction; osteoprotegerin; M-CSF

The etiology of Rheumatoid arthritis (RA) is still unknown, although the discovery of a receptor activator of the NF-κB ligand (RANKL) and osteoprotegerin (OPG), which is a decoy receptor of RANKL, accelerates understanding of the mechanism underlying bone formation and destruction.1–3) Inflammation and the immune reaction in the synovial membrane is followed by the infiltration of inflammatory cells, synovial cell proliferation, pannus formation, and bone erosion and destruction in affected joints. Bone destruction in RA patients is considered to be the consequence of chronic synovial inflammation. However, bone destruction is not able to begin, unless osteoclasts are formed and activated. In brief, inflammation and bone destruction may occur uncoupled.4) Therefore, therapeutic agents developed for the suppression of osteoclasts, in addition to anti-inflammatory and immunosuppressant activity, will be useful and indispensable for RA therapy. Currently, most treatments are directed to correct the immune aberration and to reduce inflammatory mediators. As therapeutic agents, disease modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and steroids are clinically common, and recently, TNF-α-neutralizing therapy has provided a sustained clinical benefit.5) However, the use of DMARDs has been impeded by the existence of a large number of non-responders and by gravely adverse effects with a high frequency.6) Therapy using the antibody against TNF-α receptor or TNF-α itself entails a high cost, hypersensitivity to medications, and infection due to TNF-α blockade, which raise barriers against clinical use.7,8) Thus, the validity of long-term treatment with these medicines has not yet been proven.

So far, we have reported that some Kampo medicines (traditional Japanese herbal medicines) effectively suppress collagen-induced arthritis (CIA) or spontaneously developed arthritis in pX transgenic mice.9–11) Kampo medicine uses combination formulas and usually consists of several medicinal plants, which sometimes have analgesic, anti-inflammatory, immunomodulatory, or diuretic effects. Dai-bofu-to (DBT), a Kampo medicine, is applied to the patients who are afflicted with RA for a long time and as a result have reduced physical strength. DBT is a water extract from 15 medicinal plants and has been developed on the basis of the known functions of each of these plants. Rehmanniae Radix, Angelicae Radix, Cnidii Rhizoma and Eucommiae Cortex are known to maintain or assist blood circulation. Aconitii Tuber, Paeoniae Radix, Angelicae Radix and Glehniae Radix cum Rhizoma show analgesic effects. Astragali Radix, Ginseng Radix and Zizyphi Fructus are used as a tonic. In addition, some medicinal plants with diuretic effect, for example Atractylodis Lanceae Rhizoma and Achyranthis Radix, are included in DBT. However, there are only a few reports to clarify the efficacy and mechanism by which Kampo medicines exert their effects.12–14) To pave a way for Kampo medicines to be commonly used for the therapy of various diseases, scientific evaluation of Kampo medicine is urgently required. Therefore, in the present report we attempted to assess the efficacy of DBT as a therapeutic agent for RA using RA model mice.

MATERIALS AND METHODS

Animals Male DBA/1J mice were purchased from Nippon Charles River (Kanagawa, Japan). pX transgenic mice carrying HTLV-1 env-pX region were kindly provided by Dr. Iikwara of the University of Tokyo.15) Male pX transgenic mice were bred with female BALB/c mice, then the offspring were used after determining the presence of the pX gene. The transgene was detected through dot plot hybridization using DNA prepared from mouse tails, and littermates (BALB/c mice) were used as controls. They were housed in a temperature-controlled room (at 23 ± 1 °C) with lighting from 6 a.m. to 6 p.m. under specific-pathogen-free conditions. They were fed a sterilized commercial diet with a low protein content (Nippon Crea Co., Ltd., Shizuoka, Japan) and given water ad libitum at the Laboratory Animal Center of Nagoya City
University. All DBA/1J mice were used at 8 weeks of age, and pX transgenic mice were used at 4 weeks of age. All animal procedures were approved by the institutional animal care and use committee of Nagoya City University.

**Preparation of Dai-bofu-to (DBT)** DBT (dose per person per day) were prepared as follows. Glycyrrhizae Radix (1.5 g), Atractylodis Lanceae Rhizoma (3.0 g), Zingiberis Rhizoma (1.5 g), Paeoniae Radix (3.0 g), Zizyphi Fructus (1.5 g), Aconiti Tuber (0.5 g), Gladiolae Rhizoma cum Rhizoma (3.0 g), Angelicae Radix (3.0 g), Rheumanniae Radix (3.0 g), Cnidii Rhizoma (2.0 g), Ginseng Radix (1.5 g), Achyranthis Bidentatae Radix (1.5 g), Eucommiae Cortex (3.0 g), Notopertygii Rhizoma (1.5 g), and Astragali Radix (3.0 g) were weighed, added to 700 ml water, decocked for 1 h and concentrated to 300 ml. This decoction was lyophilized to give a powdered extract of 10.5±0.3 g.

**Induction and Clinical Evaluation of CIA** Mice were randomly separated into four groups; normal: non-immunized mice, control: untreated CIA mice, DBT: DBT-treated CIA mice, FK506: FK506-treated CIA mice. CIA was induced and evaluated as follows. Bovine type II collagen (CII) was dissolved in 0.01 M acetic acid at a concentration of 3 mg/ml and was emulsified in an equal volume of complete Freund's adjuvant (Wako, Osaka, Japan) containing 4 mg/ml and was emulsified in an equal volume of complete Freund's adjuvant (Wako, Osaka, Japan) containing 4 mg/ml and was emulsified in an equal volume of complete Freund's adjuvant (Wako, Osaka, Japan) containing 4 mg/ml.

**Preparation of Dai-bofu-to (DBT)** DBT (dose per person per day) were prepared as follows. Glycyrrhizae Radix (1.5 g), Atractylodis Lanceae Rhizoma (3.0 g), Zingiberis Rhizoma (1.5 g), Paeoniae Radix (3.0 g), Zizyphi Fructus (1.5 g), Aconiti Tuber (0.5 g), Gladiolae Rhizoma cum Rhizoma (3.0 g), Angelicae Radix (3.0 g), Rheumanniae Radix (3.0 g), Cnidii Rhizoma (2.0 g), Ginseng Radix (1.5 g), Achyranthis Bidentatae Radix (1.5 g), Eucommiae Cortex (3.0 g), Notopertygii Rhizoma (1.5 g), and Astragali Radix (3.0 g) were weighed, added to 700 ml water, decocked for 1 h and concentrated to 300 ml. This decoction was lyophilized to give a powdered extract of 10.5±0.3 g.

**Histological Assessment** The hind and front paws were fixed in 15% phosphate-buffered formalin for 3 d, decalcified in 10% EDTA for 14 d at 4 °C, then embedded in paraffin. Serial paraffin sections (7 μm) were stained with hematoxylin and eosin (H&E). Histopathological changes in joints were scored using the following parameters: 0: normal, 1: infiltration of inflammatory cells, 2: synovial hyperplasia, 3: pannus formation, 4: bone erosion, 5: bone destruction.

**FACS Analysis** Polypeptide lymph nodes were homogenized with a rough glass homogenizer to create single cell suspensions, which were then washed in Hanks balanced salt solution (HBSS). The cells (2×10^6 cells/tube) were stained for 1 h with 0.5 μg of the following antibody: PE-conjugated mAb to CD45R/B220 and CD8 and FITC-conjugated mAb to CD90 and CD4. All antibodies used in this study were obtained from PharMingen (San Diego, CA, U.S.A.). After staining, the cells were fixed with BD FACS™ Lysing solution (Becton Dickinson, Mountain View, CA, U.S.A.) for 10 min, and then washed repeatedly with HBSS. The level of non-specific staining was determined using isotype-matched Ab of irrelevant specificity. Cells were analyzed by flow cytometry on a FACSsort using Cell Quest software (Becton Dickinson, San Jose, CA, U.S.A.).

**RT-Southern Blot Analysis** Total RNA from murine joints was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instruction. The extracted RNA was treated with DNase I (Invitrogen, Carlsbad, CA, U.S.A.) in order to degrade contaminating DNA. The RNA was dissolved in DEPC-treated water and quantified by GeneQuant II (Amersham Pharma Biotech, Buckinghamshire, U.K.). To prepare first strand cDNA, 500 ng of total RNA was reverse-transcribed using Revertra Ace-α (Toyobo Co, Osaka, Japan) according to the manufacturer’s instructions. The resulting cDNA was subjected to hot-start PCR amplification with Ampli Taq polymerase (Applied Biosystems, Foster City, CA, U.S.A.) using specific PCR primers, as shown in Table 1. The number of cycles necessary to amplify cDNA but remain below saturation was determined for each primer set. Each thermal cycle of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C was applied for 15 cycles (β-actin), 24 cycles (RANK), 28 cycles (RANKL and M-CSF) and 32 cycles (OPG). PCR products were applied to 1.5% agarose gels and then transferred to positively charged nylon membranes. After fixation under ultraviolet irradiation, the membranes were hybridized with digoxigenin-labeled (DIG-labeled) cDNA probes and visualized using alkaline phosphatase-labeled anti-DIG antibody (Roche, Mannheim, Germany). The density of interesting bands was determined with a Lumi-Imager F1 (Roche, Mannheim, Germany).

**Statistical Analysis** Data was represented as the mean ± S.D. of the mean. Statistical differences were determined using Student’s t-test. A probability of less than 0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Primer Sequences Used in the Present Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANKL forward 5’-CTCTTCATGAAAACCGGAGG-3’</td>
</tr>
<tr>
<td>RANKL reverse 5’-CGACATACACCATACGACG-3’</td>
</tr>
<tr>
<td>RANK forward 5’-ATCTCTCGTGTAGTGTGCGCTG-3’</td>
</tr>
<tr>
<td>RANK reverse 5’-TGTGTGACGCTTCGAGTGTTG-3’</td>
</tr>
<tr>
<td>OPG forward 5’-TGACACACTTATACGGACAG-3’</td>
</tr>
<tr>
<td>OPG reverse 5’-TTCTCTAACTCTTCCTTGGGCCC-3’</td>
</tr>
<tr>
<td>M-CSF forward 5’-CTGGGCTTGGGAGTATGCTCC-3’</td>
</tr>
<tr>
<td>M-CSF reverse 5’-CTGGGCTTGGGAGTATGCTCC-3’</td>
</tr>
<tr>
<td>β-Actin forward 5’-AGGCCGCCTCATAGCTTTCTC-3’</td>
</tr>
<tr>
<td>β-Actin reverse 5’-GGTGGTGAGGGAGTGCATC-3’</td>
</tr>
</tbody>
</table>
mean±S.E. of the number of animals described in the legends. Statistical significance was determined by non-paired Student’s t-test, Mann–Whitney U-test, Dunnett’s test, and Turkey–Kramer test using Stat Light software. p values less than 0.05 were considered significant.

RESULTS

CIA in mice is an experimental model of autoimmune diseases induced by immunization with type II collagen (CII).16 It is also a commonly used animal model for RA, because some clinical and histological features of CIA resemble those of RA in humans.17 We therefore examined the effect of DBT on the development of CIA. DBA, which were dissolved in the daily intake of drinking water at a concentration of 10 times the daily human dose (1.72 g/kg/d), were given to the mice from three days before the first immunization to two weeks after the second immunization. On the other hand, FK506, which was used as a positive control drug, was administered orally everyday except Sunday from the first day of immunization at a dose of 10 mg/kg. Figure 1 demonstrated that DBT significantly reduced the severity of joint inflammation during the course of the experiment, and significantly delayed the onset of arthritis from 19.1 to 26.2 days after the first immunization. The incidence of arthritis reached 100% in the control and DBT-treated CIA mice. On the other hand, an immunosuppressant, FK506, suppressed arthritis more dramatically than DBT, delayed the onset more than DBT, and decreased the incidence to less than the control and DBT group. This result indicated that FK506 could be a more effective therapeutic agent for arthritis than DBT. However, FK506 treatment dramatically decreased the body weight of mice for one week after the first immunization (data not shown), which is regarded as a serious adverse effect.

The effect of DBT was also assessed by histological examination in arthritic joints 5 weeks after the first immunization. Histological changes were characterized by synovial hyperplasia, inflammatory cell infiltration of the synovial sublining layer, and bone erosion and degradation in the joints of control CIA mice (Fig. 2A). In DBT-treated CIA mice, bone erosion and degradation were scarcely detected, and inflammatory cell infiltration was also extremely reduced, although synovial hyperplasia was observed to a small extent (Fig. 2B). When histological grades were compared between the control and DBT-treated groups, DBT-treated mice showed a much lower histological score than control CIA mice (Fig. 2D). These results indicate that DBT can suppress changes in the arthritic joints.

It has been shown that both humoral and cellular immune responses to CII are involved in the pathogenesis in CIA. CIA is a T cell- and TNF-dependent disease that recapitulates several aspects of RA.18 In addition, polyclonal activation of B cells is often observed in autoimmune diseases, and anti-type II collagen (CII) antibody is detected in CIA.17 We next examined the effect of DBT on the lymphocyte population in lymph nodes. When the number of T or B cells in popliteal lymph nodes close to the arthritic joints was measured by flow cytometry, CIA induction decreased T cells and increased B cells, compared to normal mice (Fig. 3). This result suggests that polyclonal activation of B cells might occur in CIA mice, or that some type of T cells or activated T cells might be translocated from lymph nodes to the affected joints. In DBT-treated mice, the T cell number increased more than in the control CIA mice, while B cell number deceased to less than the control CIA mice. That is, DBT counteracted the changes in lymphocyte population by arthritis induction. The CD4+/CD8+ T cell ratio in popliteal lymph node T cells was not changed by arthritis induction or by DBT treatment (data not shown). When we measured the levels of anti-CII antibody in CIA mice, DBT was not able to reduce it (data not shown). This result suggests that the effect of DBT on the changes in lymphocyte population may be due mainly to suppressing the translocation of activated T cells, but not by suppressing polyclonal activation of B cells. We have so far represented that DBT effectively suppresses spontaneously developed arthritis in pX transgenic mice.10 In the present study, upon administration to pX transgenic mice, as described in Materials and Methods, DBT suppressed the severity of arthritis in pX transgenic mice (arthritis score;
Therefore, we studied the effect of DBT on lymphocyte population in popliteal lymph nodes. When the difference in the lymphocyte population was examined between pX transgenic mice and BALB/c mice that are littermates of the pX transgenic mice, arthritic pX transgenic mice possessed fewer T cells and more B cells in their joints than BALB/c mice. However, DBT treatment increased T cells and decreased B cells, resulting in a number close to the T and B cells of BALB/c mice (Fig. 4). On the other hand, DBT treatment of BALB/c mice inversely reduced T cells and increased B cells.

It is well known that the activation of osteoclasts is indispensable to bone erosion, which is observed in the joints of RA patients. Recently, osteoclast differentiation and activation has been found to be regulated by some factors such as RANKL, RANK and OPG. Especially, the balance between RANKL and OPG is important in order to ultimately determine whether osteoclastogenesis is activated or inhibited. In addition, osteoclastogenesis is also regulated immunologically by T cells. We therefore examined the effect of DBT on factors involved in osteoclast differentiation and activation. Total RNA was extracted from the joints of CIA mice 5 weeks after the first immunization, applied to RT-PCR, and then detected with cDNA probes labeled with digoxigenin. RANKL and RANK mRNA levels were increased by arthritis induction, while DBT did not influence their mRNA levels in CIA mice (Fig. 5). On the other hand, OPG mRNA lev-
els were much higher in DBT-treated CIA mice than in control CIA mice. When the balance between RANKL and OPG was considered, DBT treatment decreased the RANKL/OPG ratio. This result suggests that DBT may exert a suppressive effect on osteoclast formation. Furthermore, macrophage colony-stimulating factor (M-CSF) is well known to be an essential factor in inducing osteoclasts. As shown in Fig. 6, M-CSF mRNA levels were increased by arthritis induction, while DBT treatment suppressed the increase, suggesting that DBT may interrupt the osteoclastogenic action of M-CSF.

DISCUSSION

In the present study, the efficacy of DBT to suppress arthritis was confirmed by using an animal model for RA. It has been shown that both humoral and cellular immune responses to CII are involved in the pathogenesis of CIA. In fact, the disease can be passively transferred to naïve recipients by IgG antibodies specific for CII, and further anti-CII antibody was detected in RA patients, as well as in CIA mice and pX transgenic mice. Lymphocytes from animals immunized with CII and CII-specific T cell lines also transmit the disease. To disclose the mechanism underlying the effect of DBT, we investigated its effect on serum anti-CII antibody levels. DBA reduced the severity of CIA and significantly delayed the onset, whereas DBA did not influence serum anti-CII antibody levels (data not shown). In brief, DBA cannot afford to modify humoral immunity. On the other hand, the lymphocyte population in popliteal lymph nodes, which are close to the joints of the hind paws, was markedly changed by CIA induction or pX transgenic mice. This change was explained by the polyclonal activation of B cells and the relative reduction in T cells, or by the acceleration of T cell translocation from lymph nodes to joints and the relative increase in B cells. In addition, the ratio of CD4+/CD8+ T cell was not varied before and after CIA induction or between pX transgenic and BALB/c mice, indicating that a specific subset of T cells was not necessarily translocated to the joints. The effect of DBT on the lymphocyte population was to restore the changes in CIA or pX transgenic mice to normal levels. Considering the fact that DBT does not affect humoral immunity, DBT may be likely
to modify T cell functions, including T cell activation and translocation. Although the precise effect of DBT on immunity was not determined, its modulatory effect on cellular immune response may play a role in suppressing the development of arthritis.

DBT consists of 15 medicinal plants, some of which are reported to show anti-allergy effects. Therefore, an immunosuppressive effect on T cells might be expected in such medicinal plants. However, concerning the immunosuppressive effect of the constituent crude drugs of DBT, there is only a report that some phenethyl alcohol glycosides isolated from Rehmanniae radix have immunosuppressive activity in inhibiting hemolytic plaque-forming cells.26) Thus, further phytochemical study of DBT is required to clarify what ingredient represents the activity of DBA.

Bone remodeling and homeostasis is an essential function that regulates skeletal integrity. However, profound focal and generalized bone loss attributable to the cellular action of osteoclasts is observed in RA patients.27) Therefore, the suppression of osteoclast differentiation and activation could be beneficial for RA treatment. Osteoclasts differentiate from osteoclast progenitor cells by two uniquely essential signals provided by osteoblasts. One is mediated by M-CSF through its cognate receptor c-fms, expressed on osteoclast progenitor cells.28) The other is transmitted by RANKL through RANK expressed on osteoclast progenitor cells, mature osteoclasts, and chondrocytes. Thus, M-CSF and RANKL are together essential and sufficient for osteoclast production, and promote the multinucleation of pre-fusion osteoclasts and the survival of nascent osteoclasts. On the other hand, OPG, a decoy receptor of RANKL, is known to reduce osteoclast survival of nascent osteoclasts.29) Thus, further phytochemical study of DBT is required to clarify what ingredient represents the activity of DBA.

REFERENCES

11) Ono Y., Inoue M., Mizukuni H., Ogihara Y., Manuscript is submitted.