Suppressive Effect of Kanzo-bushi-to, a Kampo Medicine, on Collagen-Induced Arthritis

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Kanzo-bushi-to (KBT) is a traditional Japanese herbal medicine (Kampo medicine), which is used in Japan to treat rheumatoid arthritis. In the present study, we investigated the suppressive effect of KBT on collagen-induced arthritis (CIA) and further studied the underlying mechanism. CIA was induced in male DBA/1J mice by immunization with bovine type II collagen, followed by a booster injection 21 d later. KBT was given at a dose of 430 mg/kg/d from three days before the first immunization to the end of the experiment. KBT suppressed CIA development effectively and further protected focal bone bone erosion and bone destruction as evidenced by the reduced histological score. Histochemical examination revealed that KBT decreased TRAP-positive cells at the synovium-bone interface and at the sites of focal bone erosion, coincident with the findings that RANKL/OPG mRNA ratio was significantly reduced by KBT treatment. KBT also decreased mRNA levels of M-CSF and iNOS in joints and of INOS in peritoneal macrophages. In conclusion, KBT prevented osteoclast generation by decreasing RANKL/OPG ratio and M-CSF mRNA levels, resulting in reduction in bone erosion and destruction. In addition, KBT has anti-inflammatory effect such as the suppression of iNOS expression in peritoneal macrophages and joints of CIA mice. These findings suggest that KBT is a potential new therapeutic agent for the treatment of RA.

Key words Kanzo-bushi-to; Kampo medicine; collagen-induced arthritis; bone destruction; osteoclast

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by acute arthritis affecting several joints and accompanying synovial hyperplasia, ultimately leading to joint destruction and deformity, which reduce the quality of life severely. Recently, the mechanism underlying the bone remodeling and bone loss has become increasingly clear, although the etiology and pathogenesis of RA have not yet been fully understood. The presence of osteoclasts at sites of focal bone erosion in RA and in animal models of arthritis suggests that osteotropic factors leading to osteoclast differentiation and activation may play a critical role in the pathogenesis of the erosion in joints affected with RA. Several lines of evidence indicate that some cytokines and hormones including macrophage colony-stimulating factor (M-CSF), transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1α, IL-6, IL-7, IL-11, IL-12, IL-17, calcitonin, estrogen regulate osteoclast differentiation and activation. In addition, an essential factor for osteoclast differentiation, receptor activator of nuclear factor-κB ligand (RANKL), has recently been identified and demonstrated to play a critical role in the pathogenesis of bone erosion in inflammatory arthritis. A decoy receptor of RANKL, osteoprotegerin (OPG), was also identified and found to suppress bone resorption associated with osteoclast development. Collagen-induced arthritis (CIA) is an experimental model for RA, and has many morphological features similar to those of human RA, including synovitis, pannus formation, and erosion of cartilage and bone. The expression of RANKL, RANK, and OPG in the CIA model has been demonstrated to play an important role in developing osteolytic lesions in local subarticular bone as well as in periarticular osteopenia and systemic osteoporosis. These findings suggest that the RANKL-RANK signaling pathway and the factors involved in the regulation of RANKL, RANK, and OPG expression can be novel targets for the treatment of RA to protect joints from bone destruction.

Important issues concerning RA therapy are the ability to control symptoms and signs of the disease for prolonged periods as well as the capacity to retard the damaging effect of rheumatoid inflammation on articular cartilage and bone. For the remedy of RA, disease modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and steroids are clinically common therapeutic agents, and recently TNF-α-neutralizing therapy in combination with methotrexate provided sustained clinical benefit. However, the validity for long-term treatment with these medicines has not yet been proven, and further adverse events were reported with extremely high frequency, which limits their use early in the disease process and interfere with prolonged administration. Rheumatoid bone destruction, attributed to activated synovocytes and bone-resorbing osteoclasts, can not be easily controlled by inhibiting only one of the factors involved in the multiple pathological process. Taking into consideration the state of RA therapy and the intricate pathogenesis of RA, the combination therapy using plural therapeutic agents or the therapy using agents containing plural constituents may be useful to suppress inflammatory arthritis.

Traditional Japanese herbal medicines (Kampo medicines) usually consist of several medicinal plants, and are applied to chronic diseases depending on the degree of development of the disease and the condition of the patient. We have so far tested the effectiveness of eight Kampo medicines against arthritis model mice and found that four Kampo medicines (Makyo-yokkan-to, Dai-bofu-to, Keishi-ka-jutsubu-to, and Kanzo-bushi-to) effectively suppressed the severity of arthritis.
tis in an induced-arthritis model, CIA mice.\textsuperscript{25} Especially, KBT decreased serum anti-type II collagen antibody levels significantly,\textsuperscript{25} although such an effect was not seen in other Kampo medicines. These results suggest that the inhibition of arthritis by KBT is ascribed to anti-inflammatory and immunosuppressive effect. We therefore evaluated the effect of KBT on bone destruction in the joints of CIA model mice, as one of the most important aims of RA therapy is to block bone destruction. We provided here several lines of evidence that KBT effectively suppressed bone destruction in arthritic joints, accompanying the reduction of osteotropic factors.

MATERIALS AND METHODS

Animals Male DBA/1J mice were purchased from Nippon Charles River (Kanagawa, Japan). All mice were kept in a temperature-controlled room (23±1°C) with lighting from 6 a.m. to 6 p.m., under specific-pathogen-free conditions and given a sterilized commercial diet (CE-2; Nippon Crea Co., Ltd., Shizuoka, Japan) and water ad libitum at the Laboratory Animal Center of Nagoya City University. Mice were used at 8 weeks of age. All animal procedures were approved by the institutional animal care and use committee of Nagoya City University.

Preparation of Kanzo-bushi-to (KBT) KBT (dose per person per day) was prepared as follows. Cinnamomi Cortex (3 g; Tsumura Co. Ltd., Lot No. 18054631), Glycyrrhizae Radix (2 g; Tsumura Co. Ltd., Lot No. 19002891), Atractylodis Lanceae Rhizoma (4 g; Daiko Galenical Co., Ltd., Lot No. OL12), Aconiti Tuber (0.5 g; Mikuni Pharmaceutical Industrial Co., Ltd., Lot No. C558) were added to 700 ml water, decocoted for 1 h and concentrated to 300 ml. This detection was filtrated through cheese-cloth and lyophilized to give 2.7±0.2 g of powdered extract. Main ingredients are cinnamic aldehyde, glycyrrhizin, atractyl, and aconitine in Cinnamomi Cortex, Glycyrrhiza Radix, Atractylodis Lanceae Rhizoma, Aconiti Tuber, respectively.

Induction of Collagen-Induced Arthritis (CIA) in Mice Mice were randomly separated into three groups: normal, non-immunized mice; control, untreated CIA mice; KBT-treated, KBT-treated CIA mice. CIA was induced and evaluated as described previously.\textsuperscript{25} Briefly, the severity of arthritis was evaluated for each paw by scoring method according to the degree of inflammation, where grade 0, normal; grade 1, swelling of one finger; grade 2, swelling of more than two fingers; grade 3, swelling of heel; and grade 4, joint deformity with ankylosis, resulting in maximum score of 16 per animal.

Polymerase Chain Reaction Amplification of Reverse-Transcribed mRNA For semiquantitative reverse-transcriptase PCR (RT-PCR) analysis, total RNA from peritoneal macrophages was extracted using Trizol reagent (Invitrogen, Carisbad, CA, U.S.A.) according to the manufacturer’s instructions. The extracted RNA was treated with DNaseI (In- dustrial Co., Ltd., Lot No. C358) were added to 700 ml polymerase (Applied Biosystems, Foster City, CA, U.S.A.) using specific PCR primers for iNOS and β-actin as shown in Table 1. PCR products were analysed on 1.5% agarose gels stained with ethidium bromide.

RT-southern Blot Analysis Total RNA from murine joints was isolated using Trizol reagent and first strand cDNA was prepared as described above. 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 and cell type. Each thermal cycle of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C was applied for 15 cycles (β-actin), 24 cycles (RANK), 25 cycles (IL-6), 28 cycles (RANKL and M-CSF), 32 cycles (OPG), and 35 cycles (iNOS and TNF-α). 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 Lumi-Imager F1 (Roche, Mannheim, Germany).

Histological Assessment 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), and with fast red violet for tartrate-resistant acid phosphatase (TRAP) activity. TRAP staining was performed according to the manufacturer’s instructions attached to the kit (Muto Chemical Co., Ltd, Tokyo, Japan). 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.

Assay for Nitric Oxide Produced by Peritoneal Macrophages Thioglycolate-elicited peritoneal macrophages were prepared as described previously with a minor modification. Briefly, DBA/1J mice were injected intraperi-
toneally with 2 ml of 3% thioglycolate medium (Difco Laboratories, Detroit, MI, U.S.A.) one day after second immunization in CIA model. Six days later, peritoneal macrophages were harvested from the abdominal cavity and maintained in RPMI1640 (IrvineScientific Co, Santa Ana, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS). The resulting macrophages were seeded at a concentration of $2 \times 10^6$ cells/ml and incubated in the presence or absence of lipopolysaccharide (LPS: Sigma, St. Louis, MO, U.S.A.) 1 µg/ml and IFN-γ (Pepro Tech, Inc, London, U.K.) 100 U/ml for 20 h. Nitric oxide (NO) production was determined by measuring as nitrite concentration in the culture medium using Griess reagent.26

Western Blot Analysis Following 24 h incubation in the presence or absence of LPS (1 µg/ml) and IFN-γ (100 U/ml), thioglycolate-elicited peritoneal macrophages were rinsed with PBS, and then lysed in the lysis buffer containing 20 mM Tris/HCl pH 8.0/137 mM NaCl/2 mM EDTA/1% glycerol/1% Triton-X-100 on ice. The resulting cell lysate (20 µg of protein) was subjected to 8% SDS-PAGE analysis. After electrophoresis, the separated proteins were transferred to immobilon-P transfer membrane (Millipore Co, Bedford, MA, U.S.A.) with a wet electrotransfer system (Bio-Rad, Richmond, CA, U.S.A.). The membranes were incubated with blocking buffer (Tris–borate–EDTA buffer (TBS), pH 7.5/5% powdered skimmed milk/1% glycerol/1% Triton-X-100) for 1 h and incubated with a 1 : 3000 dilution of anti-iNOS antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, U.S.A.) in TBS, pH 7.5/1% powdered skimmed milk at 4 ºC overnight. The membranes were washed in three changes of wash buffer (0.05% Tween-20 in TBS), and then incubated with a 1 : 3000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG antibody (Bio Lad, Hercules, CA, U.S.A.) at 4 ºC overnight. Finally, they were washed in four changes of the wash buffer, and iNOS was detected using CDP-Star (PE Biosystems, Bedford, MS, U.S.A.) as a substrate of alkaline phosphatase. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, U.S.A.).

Statistical Analysis Data were represented as mean±S.E. of the number of animals described in the legends. Statistical significance was determined by non-paired Student's $t$-test, or Mann–Whitney $U$-test using Stat View software. $p$ values less than 0.05 were considered significant.

RESULTS

Suppressive Effect of KBT on the Development of CIA

We first determined the effect of KBT on the development of arthritis using CIA model mice. KBT was administered at a dose of 0.43 g/kg/d from 3 d before the first immunization with bovine type II collagen until the end of the experiment. The dose of KBT corresponds to a concentration of 10 times the daily human dose and suppressed macrophage function effectively in ex vivo study in our previous study.25 Figure 1 shows that KBT treatment reduced the severity of joint inflammation significantly and delayed the onset of arthritis from 21.8 to 26.8 d after the first immunization. The incidence of arthritis reached to 100% in the end of experiment and did not vary between KBT-treated and control CIA groups. During the course of the experiment, the change in body weight and other changes in the appearance were not observed in KBT-treated group. Although data were not shown, immunosuppressant, FK506 (10 mg/kg/d, p.o.), markedly reduced the arthritis severity (average score: 2.0±0.2 at day 36), and prolonged the onset to 29.3 d. However, FK506 decreased the body weight of mice severely, which was considered as an adverse effect.

Reduction of Joint Destruction by KBT Five weeks after the first immunization, the tarsocural joints of KBT-treated, control and normal mice were examined histologically after H&E staining of joint sections. No synovitis, pannus formation, or focal bone erosion was seen in normal mice joints (Fig. 2C), while in control CIA mice infiltrating inflammatory cells were observed in the hyperplastic synovium, and periartthritis, osteomyelitis, and focal bone erosion was prominent (Fig. 2A). In KBT-treated CIA mice, the severity of arthritis was markedly ameliorated, although the hyperplasia of synovium and focal bone erosion were seen in their joints (Fig. 2B). When the inflammation in joints was assessed by histological scoring, as described in Materials and Methods, KBT decreased the histological score significantly, relative to control CIA group (Fig. 2D).

It is well known that osteoclasts play a critical role in bone resorption and can be observed at the sites of bone erosion in RA patients. We therefore stained the sections of tarsocural joint with TRAP, which stains tartrate-resistant acid phosphatase-positive cells. In control CIA mice, TRAP-positive cells were seen at the sites of focal bone erosion (Fig. 3A) and within erosive pits in the bone (data not shown). On the
other hand, TRAP-positive cells were extensively reduced in KBT-treated mice, coincident with the decrease in bone erosion (Figs. 3B, C).

**Expression of RANKL, RANK, and OPG** In view of the evidence that RANKL, RANK, and OPG are critically involved in osteoclast differentiation and activation, we evaluated the effect of KBT on their expression in affected joints by determining their mRNA expression. 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 (RT-southern analysis). RANKL and RANK mRNA levels were higher in the joints of CIA mice than those of normal mice, while their expression was suppressed by KBT treatment (Figs. 4A, B). This result was supported by the evidence that the decreased expression of RANKL and RANK was observed in the joints of KBT-treated CIA mice as detected by immunohistochemistry (data not shown). On the other hand, although the ex-
Fig. 4. Effect of KBT on RANKL, RANK, and OPG mRNA Expression in Arthritic Joints of CIA Mice

Total RNA was extracted from the joints of carpal bones and digital bones of hands on day 14 after the second immunization. The mRNA was reverse-transcribed, amplified using respective specific primers for RANKL, RANK, and OPG, and detected with digoxigenin-labeled cDNA probes. Each band shown in Fig. 4A represents a mouse. Densitometric quantitative analysis of the bands in Fig. 4A was performed in Fig. 4B. The ratio of RANKL to OPG mRNA levels was represented in Fig. 4C. Values are mean ± S.E.M. of 6 mice. Statistical significance was evaluated by Student’s t-test.

Fig. 5. Effect of KBT on mRNA Expression of Cytokines Involved in Bone-Resorbing in Arthritic Joints of CIA Mice

Total RNA was extracted from the joints of carpal bones and digital bones of hands on day 14 after the second immunization. The mRNA was reverse-transcribed, amplified using respective specific primers for TNF-α, IL-6, and M-CSF and detected with digoxigenin-labeled cDNA probes. Each band shown in Fig. 5A represents a mouse. Densitometric quantitative analysis of the bands in Fig. 5A was performed in Fig. 5B. Values are mean ± S.E.M. of 6 mice. Statistical significance was evaluated by Student’s t-test.
pression of OPG was slightly enhanced by induction of CIA, it was not influenced by KBT treatment. Considering the importance of RANKL/OPG balance in osteoclast development, the significant decrease in RANKL/OPG ratio after KBT treatment indicated that KBT effectively suppressed osteoclast formation (Fig. 4C).

Effect of KBT on Osteotropic Factors A number of osteotropic factors induces osteoclastogenesis by enhancing osteoclast differentiation, activation, or survival. Among them, some cytokines such as TNF-α, M-CSF, and IL-6 were intimately implicated in joint destruction of RA. We therefore assessed the effect of KBT on the mRNA expression of osteotropic factors in affected joints. In control CIA mice, all the cytokines that we studied were markedly induced by CIA compared with normal mice (Fig. 5A). When the effect of KBT was examined, KBT markedly suppressed mRNA expression of M-CSF, in addition to slight inhibition of IL-6 mRNA expression. However, TNF-α mRNA levels were not significantly affected by KBT treatment (Fig. 5B).

Inhibition of NO Production and NO Synthase (iNOS) Expression in Peripheral Macrophages and Joints of CIA Mice To study the inflammatory effect of KBT, thioglycolate-elicited macrophages, which were prepared from CIA mice, were stimulated with LPS/IFN-γ and then NO production, iNOS, and iNOS mRNA were determined by Griess reagent method, western blot analysis, and semiquantitative RT-PCR, respectively. As shown in Fig. 6A, KBT suppressed NO production significantly, resulting from the decrease in iNOS protein (Fig. 6B) and mRNA expression (Fig. 6C). In addition, when mRNA expression in the affected joints of CIA mice was examined with RT-southern method, iNOS mRNA expression was markedly reduced in KBT-treated mice (Figs. 6D, E).

DISCUSSION

Bone destruction in RA patients is considered to be the consequence of chronic synovial inflammation. However, bone destruction is not able to start, unless osteoclasts are formed and activated, evidenced by that c-fos-deficient mice, which completely lack osteoclasts, were fully protected against bone destruction despite the presence of synovial inflammation. There is ample evidence that joint inflammation and destruction can be uncoupled. The balance of destruction and protective mediators determines the relative erosive nature of a given arthritis, rather than the bulk of the inflammation mass. Therefore, in addition to the use of anti-inflammatory therapy, suppression of osteoclast differentiation and activation could be beneficial for the treatment of RA. Osteoclasts differentiate from osteoclast progenitor cells by two uniquely essential sigmals provided by osteoblasts. One is mediated by M-CSF through its cognate receptor c-
fms, expressed on osteoclast progenitor cells. 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 multinucleation of pre-fusion osteoclasts and survival of nascent osteoclasts. On the other hand, OPG, a decoy receptor of RANKL, is known to reduce osteoclast numbers and prevent bone erosion in CIA. OPG is steadily expressed in normal joints and is induced by TGF-β, IL-1α, and TNF-α, which are produced in inflamed joints. This suggests that the balance between RANKL and OPG is important to ultimately determine whether osteoclastogenesis is activated or inhibited. In the present study, we found that KBT reduced RANKL expression detected by RT-southern analysis and immunohistochemical study (data not shown), and that KBT also diminished M-CSF mRNA levels in joints affected by CIA. In contrast, we did not detect the change in OPG expression between control CIA and KBT-treated CIA mice. These results suggest that KBT treatment interferes with not only RANKL/RANK, but also M-CSF/c-fms signaling pathways, finally leading to the reduction in osteoclast formation. RANKL is expressed on osteoblasts, activated T cells, and synovial fibroblasts at the sites of bone erosion and synovial bone interface in inflamed joints, and is known to be regulated by many osteotropic factors. M-CSF is also produced by osteoblasts/stroma cells in inflamed joints. Taken together, the effect of KBT may be in part due to decrease in osteotropic factor production, abrogation of signal transduction of osteotropic factors in osteoblasts to induce RANKL, or suppression of T cell activation.

Several lines of evidence suggest that RANKL and RANK interaction is not the sole pathway that leads osteoclast progenitor cells to differentiation into osteoclasts. IL-1 induces the multinucleation and bone-resorbing activity of osteoclasts even in the absence of osteoblasts/stromal cells. TNF-α stimulates osteoclast differentiation by a mechanism independent of the RANKL–RANK interaction, although there is an opposite report that TNF-α acts directly on the osteoclast precursor to potentiate RANKL–induced osteoclastogenesis, even if basal levels of RANKL are not upregulated. However, although the pathophysiological importance of RANKL-independent pathway is presently obscure in osteoclastogenesis of RA patients, the finding that KBT did not affect TNF-α expression suggests that KBT cannot regulate RANKL-independent pathway effectively.

Inflammatory conditions such as RA, which are characterized by local osteolysis, are associated with activation of iNOS. The intimate implication of NO in the pathogenesis of arthritis was indicated by the evidence that NOS inhibitors reduce NO levels and ameliorate arthritis in streptococcal arthritis was indicated by the evidence that NOS inhibitors reduce NO levels and ameliorate arthritis in streptococcal arthritis. The intimate implication of NO in the pathogenesis of RA patients, the finding that KBT did not affect NO production by KBT is likely to suppress bone destruction indirectly. In this respect, NO is a new target for the treatment of RA, and KBT with inhibitory activity of NO production may be beneficial for the remedy of RA.

Considering a potential of KBT as a therapeutic agent for RA, KBT protects bone destruction in joints affected with CIA, to say the least, by two distinct ways. One is that KBT insulates RANKL–RANK interaction by reducing RANKL/OPG balance in osteoblasts and activated T cells, which may be caused by the blockade of signaling pathway or production of osteotropic factors. The other is that KBT may prevent the differentiation and activation of osteoclast progenitor cells to mature osteoclast by reducing production of osteotropic factors or modulating signaling pathway of RANK. In brief, these effects of KBT may be attributed to anti-inflammatory effect on osteotropic factor production and direct effect on osteoblasts. However, we should determine whether the joint-protective effect results from anti-inflammatory effect on the early stage of RA or direct effect on osteoblasts or other cells.

Traditional Japanese herbal medicine (Kampo medicine) is the representative of combination formulas, for example, KBT, which we studied in the present study, consists of four medicinal crude drugs: Cinnamomi Cortex, Glycyrrhizae Radix, Atractylodis Lanceae Rhizoma, and Aconiti Tubers. In addition, its characteristic is to have plural points of action, which are ingeniously integrated to improve the condition of patient and cure a disease. Actually, the evidence presented here indicated that KBT has at least two points of action, anti-inflammatory and anti-osteoclastogenic actions. In conclusion, although the precise mechanism by which KBT suppresses the severity of arthritic joints is now under investigation, KBT is a candidate for novel therapeutic agents of RA.

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