Rheumatoid arthritis (RA) is characterized by chronic inflammation of the synovial membrane in the joint, which leads to the progressive destruction of articular cartilage, ligament and bone. Several cytokines such as tumor necrosis factor $\alpha$ (TNF-$\alpha$), interleukin 1 $\beta$ (IL-1$\beta$), and interleukin 6 (IL-6) have been implicated in the pathological mechanisms of synovial tissue proliferation, joint destruction and programmed cell death in rheumatoid joint. In the Korean traditional medicine, *Hominis placenta* (HP) as an herbal component of herb-acupuncture has been widely used to treat chronic inflammatory diseases such as RA. To study the therapeutic effects of HP injection into the ST36 acupoint (HP herb-acupuncture) on the inflammatory responses of a subchondral region of rheumatoid joint, the polyarthritis-induced Sprague–Dawley (SD) rat was developed as a rheumatoid arthritis model by the intradermal injection of dried cells of *Mycobacterium tuberculosis* emulsified in squalene to the base of tail. After the onset stage (11 d after adjuvant injection) of polyarthritis, a fixed volume of HP extract was daily injected to Zusanli (ST36) acupoint on the rat’s leg for 2 weeks. The body weight, paw volume of the knee joint and articular index were exploited as an assessment method addressing arthritic symptoms, and the expression profiles of TNF-$\alpha$, IL-1$\beta$ and IL-6 at the subchondral bone of the joint were analyzed using an immunohistochemistry. After the treatment of arthritic rats with HP, the body weights and paw volumes of arthritic rats were almost restored to the levels of normal rats whereas the evaluation by the articular index was not remarkable. The TNF-$\alpha$, IL-1$\beta$ and IL-6 positive cells in the immunohistological sections of subchondral bone region of the joint significantly decreased in HP-treated (ST36 acupoint) arthritic group as compared with those in non-treated or HP-treated (non-acupoint) ones, which was coincident with the behavioral studies. In conclusion, the HP herb-acupuncture was found to be effective to alleviate the arthritic symptoms in adjuvant-induced arthritis rats as regards the body weight, joint appearance and the expression profiles of inflammatory cytokines.

Key words  *Hominis placenta*; herb-acupuncture; adjuvant-induced arthritis; inflammatory cytokine; subchondral bone

Rheumatoid arthritis is a chronic, systemic, and inflammatory disease which causes multiple joints in the body to be tender and swollen, and thus the joints become stiff and very sore. Despite of the prevalence and severe symptoms of the disease, no one has identified yet what exactly causes rheumatoid arthritis, and to date, there is no known remedy to completely cure the disease. In a pathogenic cascade of the rheumatoid arthritis, the synovial membrane plays an important role to secrete the inflammatory cytokines, such as TNF-$\alpha$, IL-1$\beta$ and IL-6, to signal the release of tissue-damaging enzymes responsible for the joint destruction.\(^1\)–\(^4\) In recent years, subchondral bone, a distal end of the long bone that forms the synovial joint has been reported to be another target to be destroyed once the articular cartilage is damaged in rheumatoid arthritis patients.\(^5\)–\(^6\) Although it has been known that the subchondral bone is significantly related to the degeneration of cartilage and the mediation of cartilage nutrition through previous studies on osteoarthritis, its precise etiological role in the progression of arthritic disease is unclear.\(^5\)–\(^7\) A question of whether or not the pathophysiological changes of subchondral bone precede and mainly affect the cartilage deterioration of the joint in arthritis patients has remained unresolved. It was reported that the expression of inflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$, and the tissue-damaging enzymes such as cathepsin and matrix metalloproteinase-9 were observed in the subchondral bone region as well as synovial membrane of the knee joint samples from human osteoarthritis or rheumatoid arthritis patients.\(^7\) In addition, the apoptotic changes of chondrocytes were found to be significantly apparent in the abnormal calcification of the subchondral bone in osteoarthritis.\(^6\) Nevertheless, little attention has been paid to developing a new therapy to alleviate the erosive damages of the subchondral bone as well as cartilage in rheumatoid arthritis. The current study reports here on the therapeutic effects of the herb-acupuncture in which a highly purified herbal extract is injected into a specific acupoint, using the water extracts of *Hominis placenta* (HP) as an herbal component on the subchondral bone of a murine rheumatoid arthritis model. In the traditional medicine of many Asian countries, acupuncture is considered as one of the most effective treatments especially for the diseases accompanied with the drastic pain, for instance, chronic arthritis.\(^8\)–\(^9\) There have been several studies on the effectiveness of acupuncture for chronic painful musculoskeletal disorders including arthritis, and partially the action mechanism of the acupuncture treatment, despite of a little controversy about the analgesic effect of acupuncture, for instance, methodological flaws, number or location of acupuncture point exploited, inadequate statistical power et al.\(^10\) However there has been no systematic study on the therapeutic effectiveness of herb-acupuncture as yet. In a treatment with an herb-acupuncture, a fixed amount of the herbal extracts, repeatedly distilled with water for extreme purity, is injected into a specific point (acupoint) of the body, which has been empiri-


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cally proven effective in the treatment of specific disorders.11,12) Accordingly, both medicinal benefits of acupuncture and herbal therapy can be attempted by a single treatment of herb-acupuncture. HP is a dried conception vessel (placenta) of a woman, which has been widely used to cure the disease of the kidney and to replenish vital essence and blood in the Korean traditional medicine. The ST36 acupoint (Zusanli), localized near the knee, is one of the effective acupuncture points with a wide range of medicinal effects including spasmyloytic and analgesic effects for the gastrointestinal tract and homeostatic effect in endocrine and metabolism etc., and also has been used for the treatments of aching of the knee joint and leg in the Korean traditional medicine.6—10,13) To verify the therapeutic effects of HP herb-acupuncture on arthritic disease, the immunohistological profiles of the inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in a subchondral bone of the knee joint as well as the clinical parameters of arthritis such as body weight, paw volume, and articular index were analyzed using an adjuvant-induced polyarthritic rat as a murine rheumatoid arthritis model.

MATERIALS AND METHODS

Animals Female Sprague–Dawley rats (120—130 g) were used. Upon arrival, the animals were randomly divided into several groups and housed for at least 8 d prior to experimental procedures. They were kept on a 12 h light/12 h dark cycle at 22±2 °C with food and water ad libitum.

Induction of Rat Adjuvant-Induced Arthritis The adjuvant-induced arthritis was induced as previously described.14—16) Rats were intradermally injected at the base of the tail with 1.5 mg of heat-killed Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI, U.S.A.) emulsified in 0.15 ml squalane (Sigma Chemical Co., St Louis, MO, U.S.A.). Control rats were similarly treated with vehicle only. All time points were considered relative to the induction day of adjuvant-induced arthritis, which was designated as day 0.

Experimental Groups and Hominis placentae-Acupuncture Treatments Experimental animals were divided into four groups: (1) saline-treated non-arthritic animals (CONT); (2) saline-treated arthritic animals (AIA); (3) HP-treated/ST36 acupoint arthritic animals (HP/Z); and (4) HP-treated/non-acupoint arthritic animals (HP/B). The water extracts of HP for herb-acupuncture were supplied in an ampule from the Korean Traditional Medicine Research Institute (KTRI). All animal experiments were approved by the Animal Care and Use Committee of the KTRI. For the histological analyses, the knee joints of rats were dissected, fixed in a solution of 4% formaldehyde in a phosphate buffered saline (PBS), and decalcified in a phosphate-buffered saline including 10% EDTA (pH 7.4). The samples were then dehydrated through an ethanol series and embedded in a paraffin block. A 10 μm section of each sample was prepared using a rotary microtome Finesse 325 (ThermoSandon Co., Comerio-Varese, Italy). The paw volume was expressed as a relative value to that of day 0. The articular index scores were assigned to every joint of the rat’s leg by two observers blinded to the animal groups. The scoring was rated on a scale of 0—4 for each knee joint: 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, and 4 = excess edema with joint rigidity.17) The total score for each animal was calculated as an articular index with a maximum value of 16.

Tissue Preparation The rats were killed on the 25th day after the adjuvant injection. For the RNA analyses, the hind paws of rats were harvested using a surgical blade. After removals of skin and muscle tissue, the knee joints were immediately snap-frozen in liquid nitrogen, and stored in a deep freezer (–80 °C). For the histological analyses, the knee joints of rats were dissected, fixed in a solution of 4% paraformaldehyde in a phosphate-buffered saline (PBS), and decalcified in a phosphate-buffered saline including 10% EDTA (pH 7.4). The samples were then dehydrated through an ethanol series and embedded in a paraffin block. A 10 μm section of each sample was prepared using a rotary microtome Finesse 325 (ThermoSandon Co., Inc., U.K.) and mounted on a 1-polylysine-coated slide for further study. Each slide was stained with haematoxylin and eosin (H–E) for the histological examination.

Immunohistochemistry Immunohistological staining was performed by the avidin–biotin immunoperoxidase technique using VectaStain Elite ABC kits (Vector Laboratories, Inc., CA, U.S.A.) and dianisobenzidine (DAB) as an enzyme substrate. The affinity-purified rabbit polyclonal antibodies for TNF-α, IL-1β, and IL-6 were purchased from Abcam Ltd., Cambridge, MA, U.S.A. Before staining, the sections embedded in paraffin were deparaffinized and hydrated through washing with xylene and ethanol series, respectively. The slides were then treated with a PBS solution containing 0.3% H2O2 in methanol for 30 min to quench the endogenous peroxidase. After washing in a phosphate-buffered saline (pH
7.2), the slides were incubated in the PBS solution containing 10% normal blocking goat serum and 0.1% bovine serum for 20 min at room temperature to minimize non-specific binding, followed by incubation with rabbit polyclonal antibody (primary) for 30 min at room temperature. After washing in the buffer, the slides were then incubated with a PBS-diluted solution of biotinylated secondary antibody for 30 min at room temperature. After washing in the buffer, the slides were incubated in a mixed solution of avidine and biotinylated horseradish peroxidase for 30 min. The immunoreactivities of the sections on a slide were then visualized using diaminobenzidine as a chromogen and the slides were photographed using a Zeiss Axoplan light microscope (Carl Zeiss Co. Ltd., Goetingen, Germany).

Data Analysis The data was presented as mean±S.E.M. The differences between groups were assessed by paired t-test and considered significant when p<0.05.

RESULTS

Evaluations of Body Weight, Paw Volume, and Articular Index To evaluate the arthritic progression of adjuvant-induced arthritis rat, three different parameters such as body weight, paw volume, and articular index were exploited as an apparent indicator of arthritis. It is previously known that as the arthritis proceeds, the redness and swelling of the joints and the body weight loss usually start to appear at the onset stage of arthritis.16) In our study, the arthritic joints of rats exhibited almost the same swelling pattern which started on day 11 and reached the peak on day 17 after the adjuvant injection (Figs. 1A, B). As shown in Fig. 2, the body weight of the adjuvant-induced arthritis rat started to decrease on day 11 (onset stage of arthritis) after the adjuvant injection as contrasted with the steady increase of body weight in case of the normal rat. At this stage, the adjuvant-induced arthritis rats were randomly divided into three groups such as AIA, HP/Z and HP/B, and then the HP treatments were performed in HP/Z and HP/B groups to examine the therapeutic effect of HP injection into ST36 acupoint. It was found that the body weight of adjuvant-induced arthritis rat in the HP/Z group remained unchanged after the onset stage even though the recovery of body weight was not sufficient to be compatible with the level of CONT group. The experimental results of paw volume were consistent with those of body weight as regarding the phase-dependent profiles of the parameters except that the paw volume of adjuvant-induced arthritis rat exhibited a different pattern to reach a maximum plateau on day 17 (chronic stage of arthritis) and to slightly decrease after this point (Fig. 3). The HP injection into a non-acupoint (HP/B) did not exert any significant effects on the variations of both body weight and paw volume of adjuvant-induced arthritis rat. In addition to body weight and paw volume, the articular index scores were determined regularly throughout the whole period of arthritic development because articular index is another accurate index to monitor the severity for the arthritic disease. The mean articular index score was given for each feet of adjuvant-induced arthritis rat and the sum of the scores for four foot (0—20) was used to clinically assess the arthritic disease activity. The articular index scores began to increase at the onset stage and reached a maximum level on day 17 after the adjuvant injection as in the profiles of body weight and paw volume (Fig. 4). Although the symptomatic difference, assessed by scoring articular index, between CONT and HP/Z groups was not remarkable as compared to other parameters, it was confirmed that the HP injection into the ST36 acupoint was able to significantly alleviate the pathogenic symptoms of arthritis.

Cytokine Immunohistochemistry in Subchondral Bone It has been known that the several inflammatory cytokines
such as TNF-α, IL-1β and IL-6 were implicated in the pathological mechanisms of both synovial tissue and fluid, and subchondral bone region of rheumatoid joint. In a bone remodeling process which is a cycle of bone resorption by osteoclasts and bone formation by osteoblasts, TNF-α and IL-1β are known to play a key role in stimulating osteoblast production of IL-6, a principal cytokine implicated in osteoclast differentiation and the osteoclastic bone resorption. 18,19) Therefore, we analyzed the protein expression levels of these cytokines in the knee joint of adjuvant-induced arthritis rat using immunohistochemistry. At day 25 (in the middle of chronic stage of arthritis) after the adjuvant injection, the immunoreactivities of the cytokines were analyzed using paraffin sections of the knee joints of the legs. As shown in Figs. 5B, F, J), the immunopositive cells of TNF-α, IL-1β and IL-6 significantly increased within the cellular aggregates, which were located between the thickened trabecular bones of the subchondral bone in an adjuvant-induced arthritis rat (Figs. 5A, E, I). The immunopositive cells were evenly distributed on the surface of thickened trabecular bones as well as within the bone marrow. The IL-1β is known to be closely associated with exacerbating pathology in the central and peripheral neuronal systems through inducing the production of TNF-α and the proliferation of macrophages. 20—22) It is also involved in the whole period of cartilage destruction of the joint in chronic arthritis rather than in early joint swelling, and its expression gradually increased with aging in rats.1,23) The cytokines were strongly expressed in subchondral bone region of an arthritic rat as compared to that of a non-arthritic one, whereas little signals of those cytokines were observed in the cartilage of the joint (Figs. 5B, F, J). Although it was previously reported that TNF-α was important only in an early stage of arthritic joint swelling and thus less efficient target for healing the chronic disease, particularly in a late stage, in comparison with IL-1β, it was proved from these results that TNF-α could be a significant indicator to assess the arthritic symptom even in a chronic stage of adjuvant-induced arthritis.1,21) The multifunctional cytokine, IL-6 was known to be related with the immune, hematopoietic, nervous, cardiovascular, and endocrine systems, as well as the bone metabolism, inflammation, and acute phase response. 24—26) Thereby, this cytokine plays pivotal roles in the pathogenesis of a variety of chronic diseases including rheumatoid arthritis. As shown in Fig. 5J, IL-6 was also highly expressed in the knee joints of the legs in an adjuvant-induced arthritis group as expected. In HP-treated/ST36 acupoint arthritis group (HP/Z), the expression of these inflammatory cytokines drastically reduced, which was precisely coincident with the results of apparent parameters such as body weight, paw volume, and articular index (Figs. 5C, G, K). To exclude the possibility that the therapeutic effects of HP herb-acupuncture could be attributed to the only intradermal injection of HP extract, that is, the pharmacological action of HP irrespective of acupuncture treatment, an equal amount of HP was injected into a non-acupoint which was randomly selected on the base of the tail. As shown in Figs. 5D, H, L, the immunohistological results of HP-treated/non-acupoint arthritic group (HP/B) did not exhibit any significant decrease in the expression of those cytokines.

DISCUSSION

Rheumatoid arthritis is characterized by chronic inflammation of the synovial membrane in the joint, which eventually leads to the progressive destruction of articular cartilage, ligament, bone, and even subchondral region. Although it has become reasonable that erosive changes of the cartilage and bones of the joint were not definitely accompanied by the preceding joint inflammation, several inflammatory cytokines were the obvious signals to activate the secretion of the tissue-damaging hydrolytic enzymes. 7,27) The various kinds of cytokines have been identified in the articular joint with arthritic disease. Of these, TNF-α, IL-1β and IL-6 are known to be the primary cytokines to mediate the marked destruction of cartilage and bone in arthritis, and have thus been investigated as an important biological target to develop the drugs for use in the treatment of active rheumatoid arthritis. 2,28,29) Thus far, only two drugs targeting TNF-α were developed and licensed to clinically treat rheumatoid arthritis.
patients, in a form of modified protein of soluble TNF receptor or monoclonal antibody to TNF-α. Our results also indicated that those cytokines were significantly secreted in arthritic joints of the legs, which means that the inflammatory cells, mainly macrophages, were infiltrated into the arthritic joints as the arthritis proceeded. Because the inflammatory cytokines such as TNF-α and IL-1β were specifically stained on the bone section, the immunopositive cells could indirectly represent the inflammatory cells infiltrated. Once the autoimmune responses are initiated by the antigen that may originate from the infectious channels outside the body, the several proinflammatory cytokines are secreted from the macrophages and synovial fibroblasts to induce the release of the destructive enzymes from macrophages, synovial fibroblasts, and chondrocytes into the synovial fluids. Thereby, the first pathological changes in rheumatoid arthritis are known to usually begin with an alteration of synovial tissue, for instance, the cell proliferation of synovium, multiplication of synovial lining cells, and edema and pannus formations etc. Besides synovial tissue, much evidence has been recently given to the identification of the etiological roles of the subchondral bone of the joint in a degenerative process of arthritis. Although it has not been clear yet whether the structural modification and erosion of the subchondral bone region were preceded or followed by the cartilage deterioration of arthritic joint, the subchondral bone in arthritis was characterized by the structural alterations, such as the increased plate thickness and density, the cellular infiltration from subchondral bone, and the increase in the number of mononuclear cells in bone marrow blood. In our results, the severe structural changes, such as the pannus invasion into the cartilage, the thickened trabecular bones, and the cellular aggregation in subchondral bone region of adjuvant-induced arthritis rat were evidently observed in arthritic samples by using H–E staining (data not shown). Thus, the pathophysiological changes of the subchondral bone have lately attracted considerable attention to assess the arthritic disease because this region might be an etiological origin to destroy the articular cartilage as well as a target to be destroyed by an inflammatory attack. In this study, we chose the
Zusanli (ST36) single acupoint to treat adjuvant-induced arthritis using HP injection even though it has been known that the other acupoints such as Xuanzhong (GB39), Yanglingquan (GB34) and Dashu (UB11) are very effective and thus a combination of these acupoints exerts more powerful effects than any single acupoint treatment to alleviate acute or chronic arthritis. However the single acupoint treatment was more appropriate to evaluate the therapeutic effectiveness of the herb-acupuncture in this study because the treatment was designed to attempt both effects of acupuncture and herbal therapies at one try. In the Chinese and Korean traditional medicine, Zusanli is one of the most frequently used and certainly the most intensively studied single acupoint in acupuncture and moxibustion therapies. Moreover, this acupoint has the advantage of relatively easy finding of the point in a small animal such as rat. In our results, HP injection into the ST36 acupoint drastically attenuated the inflammatory cytokine expression in subchondral bone region, whereas the same HP treatment to a distant non-acupoint exhibited little therapeutic effects. These results were coincident with those of previous study describing a bee venom (BV) herb-acupuncture as regards edema and nociceptive responses of Freund’s adjuvant-induced monoarthritic rat. It means that the herbal component of HP herb-acupuncture exerts little therapeutic effects unless the herbal injection is carried out only at the acupoint of the rat’s leg, as shown in the results of non-acupoint arthritic group (HP/B) (Figs. 2—4). It was also investigated whether or not the simple stimulation of acupoint ST36 free of herbal component exerted the therapeutic effects on arthritic joint. Although the arthritic symptoms were a little alleviated by simple acupuncturing when being assessed by paw volume and articular index, the remedial effectiveness was not as remarkable as the treatment by the herb-acupuncture (data not shown). As shown in Fig. 1, the joints of forefeet were also inflamed almost equally to those of legs. Although the forefeet left untreated, the arthritic symptoms of forefeet were getting better as much as the legs were by the HP treatment to both legs. These results imply that the medicinal efficacy of both herbal component and acupuncture in an herb-acupuncture are synergistic, that is, any individual treatment is not sufficiently functional in curing the disease. In conclusion, this study demonstrated that HP injection into the ST36 acupoint was highly effective to heal the chronic arthritis in comparison with HP injection into a non-acupoint or a simple acupuncture into an acupoint without the HP injection. The further development of the HP herb-acupuncture as regarding the treatment time, treatment period, and HP dosage effect may result in the more efficient therapeutic model to treat the rheumatoid arthritis patients.

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