Inhibitory Effects of Transforming Growth Factor and Drynariae Rhizoma on Leukocytosis Associated with the Chronic Phase of Arthritis in Mice

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Introduction

From ancient times in China, Korea and Japan, women who have had low back pain in climacteric and senescent periods have been treated with oriental medicines. For example, several formula have been used in treating ovary function failure, used low back pain during the climacteric period, and also used after oophorectomies because of malignant tumors. However, no reports are available as to the recovery of bone mass by any of these oriental medicines.

Drynariae Rhizoma (DR) is known to be effective for the treatment of deficient kidney manifested as lower back pain, weakness of the legs, tinnitus or toothache by tonifying the kidney, invigorating the blood and stopping the bleeding in the Korean and Chinese medicinal literature. Since a large decrease in bone mass occurs in the postmenopausal state, women are vulnerable to the type of osteoporosis known as postmenopausal osteoporosis. DR, named Gol-se-bo in Korea is the root of Drynariae Rhizoma (Oliv.), an herbaceous perennial plant belonging to the Drynaria Family.
has been known for a long time for its effects of cleansing blood and increasing circulation, and utilized as a valuable remedy for anemia, menstrual irregularities, and constipation in traditional Korean and Chinese medicine. To treat osteoporosis, a herbal formula containing DR is being used in Korean medicine\(^5\). Besides the illnesses discussed above, DR frequently appears in traditional prescriptions for bone and tendon injuries. For example, 56 of the 73 fracture prescriptions collected in the *Encyclopedia of Esoteric Prescriptions in Traditional Chinese Medicine* contain DR as one of the main ingredients\(^6\).

Clinical data has shown that these prescriptions have significant effects in reducing the time needed for injured bones to heal. When treated with a pasting medicine, Golsebo-Tang, mainly consisting of DR, crab shells and several pain-killing herbs, in the 112 closed fracture cases of people ranging from age 1 to 40 years, on average the patients regained health in 31.6 days on average, much shorter than the normal healing time of 8-10 weeks. Medicines prepared with water or wine stir-baking technique for oral intake also yielded similar results. X-ray images showed the formation of new bone tissue at fracture sites within 7-10 days of injury. This further proved the advantage of traditional prescriptions over conventional Western surgical treatment\(^7\).

Also, it was shown that the DR extract could prevent the development of bone loss induced by ovariectomy in rats\(^8\). DR extract was useful for preventing both postmenopausal osteoporosis and osteoporosis associated with the ovary function failure\(^9\). It was demonstrated that the interaction between PGE2 and its cell surface receptor results in activation of the PKA signaling pathway. Treatment and pretreatment of the DR extract strongly inhibited IL-1\(\beta\) mRNA transcription and so, LPS-stimulated inflammatory IL-1\(\beta\) production\(^9\). Water extract of DR has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis (RA), with satisfactory results. To date, several modern studies have shown only that DR could be effective against the syndrome occurring after whiplash injury and anemia in rabbits, and that polysaccharides and lysophosphatidylcholines are responsible for anti-ulcer and hypotensive actions, respectively. However, it is still not clear regarding the effects of DR on RA induced by type II collagen (CII) and complete Freund's adjuvant (CFA) in rodents\(^10\).

The potent immunosuppressive effects of transforming growth factor \(\beta\) (TGF\(\beta\))\(^11\), suggest that it may be valuable in the treatment of disease states characterized by aberrant functions of the immune system. TGF\(\beta\) has been shown to inhibit *in vitro* the proliferation of the immune-related cells including thymocytes, T- and B-lymphocytes, and hematopoietic progenitor cells, and production of immunoglobulins\(^12\). Since many of the immune cell functions influenced by TGF\(\beta\) are involved in the sequence of events leading to connective tissue destruction in arthritic lesions, TGF\(\beta\) may be effective for suppressing the pathogenesis for the chronic inflammatory disease. To examine the TGF\(\beta\) 1 effects on arthritis animals, TGF\(\beta\)1 has been administered to the rat into which streptococcal cell wall (SCW) fragments were injected. While the control rats showed the acute inflammation with chronic proliferative and erosive diseases, the TGF\(\beta\)1-treated rats showed a significantly reverse of the inflammation\(^13\). The chronic arthritis condition has been identified as T cell and monocyte-mediated immune responses\(^14\) and thus these responses should be modulated by TGF\(\beta\).

However, because these prescriptions for bone disease have been established in centuries by trial and error and their effects were confirmed only through repeated clinical applications, it is unclear how the herbs pharmacologically make the bone tissue to heal.
Possibly, the effects of DR on the circulation and immune systems might be to improve the nutritional supply and immunity of the injured site. Nevertheless, especially in the case of pasting medicine in contact with the injured tissue, DR is likely to have direct stimulations on bone formation. Currently, no scientific research has been done on this subject. In an earlier research to find unidentified pharmacological effects of DR, the authors investigated the specific effects of DR on arthritis by using an established arthritis animals. The present results showed that daily administration of TGFβ1 and DR reduced the acute and chronic phases of diseases.

Materials and Methods

1. Drynariae Rhizoma (DR), materials and chemicals

DR was kindly supplied by the Oriental Medical Hospital of Dongguk University (Kyungju, Korea). Its identity was confirmed by comparison with the descriptions of characteristics and appropriate monograph in Korea Pharmacopoeia4). The traditional method for the clinical preparation of herbal medicine was employed. Briefly, finely cut DR root of 10 g was added to distilled water (100 ml) in a flask with a condensation apparatus on the top allowing evaporated steam to reenter the system and heated at 100°C for 24 h in an oil bath, using an electric hot plate as a heat source. After the solution cooled, residue precipitation was filtered off and put into water for secondary extraction. The aqueous extracts were mixed and evaporated to dryness under reduced pressure with a rotary evaporator at 40°C. The dried residue was dissolved in the distilled water and 1% DR aqueous extract was used for cell culture.

All chemicals and laboratory materials were from Sigma (St. Louis, MO) or Gibco BRL (Grand Island, NY) unless otherwise stated. Tissue culture media and reagents, fetal bovine serum (FBS) were from Gibco (Chagrin Falls, OH). Human osteoprecursor cells (OPC-1) were obtained as described by Winn et al.16.

2. Reagents and animals

Lewis female rats were purchased from Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). They were allowed at least 1 week to adapt to the environment (25 ± 3°C, 55 ± 5% humidity and a 12 h light/dark cycle) and were used at 7 weeks of age.

Radiochemicals were from Amersham International Co. (Seoul, Korea). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea). TGFβ1 were from R&D Systems (Funakoshi, Co., Ltd., Tokyo, Japan). Streptococcal cell wall (SCW) was obtained from Biomedia (Foster City, CA).

3. Arthritis induction and TGFβ1 and DR administration

Specific pathogen free Lewis female rats (100 g) were injected with peptidoglycan-polysaccharide fragments (30 μg rhamnose/g body mass) derived from group A SCW to induce an erosive polyarthritis as previously described13). The arthritic response was quantified by determining the articular index (AI). Each of the four distal joints was scored blinded on a scale of 0-4 on the basis of swelling, redness, and degree of deformity of normal contours. The individual scores were summed to get the whole animal score with a possible maximum of 16. AI were averaged for each group of animals and reported as average ± SEM, unless otherwise indicated.

TGFβ1 and DR were intraperitoneally injected daily
for intervals specified for each experiment, up to 7 days. The TGFβ1 stock was diluted in vehicle of BSA in PBS (1 mg/ml) to 0.05-1.0 μg TGFβ1/ml vehicle immediately before intraperitoneal administration. Control animals received an equal volume (2 ml) of either the vehicle or PBS. The vehicle was found to contain < 20 pg/ml endotoxin (limit of detection)17).

4. Cell cultures and proliferation assay

At selected intervals, blood smears, hematocrits, and total white cell counts (Coulter counter, Tokyo Rika Co., Tokyo, Japan) were obtained for each animal. At the time of tissue harvest, PBMCs were isolated from heparinized blood by density gradient centrifugation through Histopaque 1083 (Sigma Chemical Co.). Proliferation was assessed in the presence or absence of stimuli, ConA (Boheringer Mannheim) and PHA (BM), as previously described14,18). After 68 h of culture, the cells were pulsed for 4 h with 0.5 μCi/well of [3H]thymidine ([3H]TdR, specific activity 6.7 Ci/mmol) (Amersham). The cultures were harvested using an automated harvester and the amount of incorporated radioactivity was determined in a liquid scintillation counter (Beta-ray counter, Beckman, USA).

5. Analytical methods

Protein contents were determined by a Protein assay kit of Bio-Rad Laboratories (Richmond, CA, USA).

6. Statistics

Results of the above animal studies are given as mean ± standard error of the mean (SEM) with groups consisting of three to six animals. The significance of difference between the two groups were also evaluated by a student’s t-test.

Results

1. Suppression of acute and chronic arthritis by TGFβ and DR

To assess the effects of TGFβ and DR as therapeutic agents for arthritis, TGFβ1 was administrated daily to Lewis rats at dosages of 0.1, 0.2, 1.0 and 2 μg/rat, and DR was administrated daily at dosages of 10, 20, 50 and 100 μg/rat, beginning 1 day before the injection of Streptococcal cell wall (SCW), which initiated the arthritis. SCW-treated rats, which did not receive TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B). However, when 1 and 2 μg of TGFβ1 and DR, displayed the acute and chronic swelling and deformity, which is typical in the SCW-induced arthritis (Fig. 1A and 1B).

The striking diminution of the acute and chronic components of the developing SCW-induced polyarthritis was not only observed in animals that received 0.1-2.0 μg TGFβ daily, but also in animals that received 10-50 μg DR daily. These animals displayed a minimal joint inflammation during the acute phase of the arthritis and during the chronic phase as well. Control animals not receiving SCW, but dosed intraperitoneally daily with TGFβ (2 μg/animal), with DR (50 μg/animal), vehicle (1 mg RSA/ml PBS), or PBS had no synovial pathology. (Data not shown)

2. Suppression of established arthritis by TGFβ and DR

Because of the profound effect of TGFβ and DR on
When administration was begun before the onset of detectable inflammation, it was next investigated whether TGFβ1 and DR could suppress the established chronic inflammatory events. TGFβ1 administration (2 μg/rat per day) was begun on day 5 for a group of SCW-injected animals and continued until day 14 (Fig. 2). Before this point, all animals had similar articular index (AI) scores. However, once daily administration of TGFβ1 was begun, the scores of the treated group decreased as compared with that of the untreated group. TGFβ1 effectively suppressed the chronic phase of the arthritis. On day 5, the AI score of the untreated group was 7.1 ± 0.4 while that of the TGFβ1 treated group was 5.2 ± 0.3 (p<0.05) (Fig. 2).

Additional studies were performed to determine when TGFβ1 must be administered to avert the acute and chronic inflammation. One study examined the effect of TGFβ1 on arthritic lesions after connective tissue destruction was already apparent. When daily
injections of TGFβ1 were begun on day 7, well into the chronic destructive phase, no significant change occurred in the AI of the animals (AI=6.3 ± 1.4 for SCW-treated animals vs. AI=5.3 ± 1.0 for SCW+TGFβ1-treated animals at day 7). Furthermore, a single injection of TGFβ1 1 day before SCW administration did not diminish the acute or chronic phases of the arthritis (AI=5.8 ± 0.6 for SCW+TGFβ1 animals; day 7; data not shown).

In another study, DR administration (10 μg/kg rat per day) was also begun on day 5 for SCW-injected animals and continued until day 14 (Fig. 3). Before day 5, all animals had similar AI scores. However, once daily administration of DR was begun daily, the scores of the DR-treated group decreased as compared with that of the untreated group. DR effectively suppressed the chronic phase of the arthritis. On day 7, the AI score of the untreated group was 6.5 ± 0.6 while that of the DR treated group was 4.6 ± 0.4 (p<0.05).

Additional studies were performed to determine when DR must be administered to avert the acute and chronic inflammation. One study examined the effect of DR on arthritic lesions after connective tissue destruction was already apparent. When daily injections of DR were begun on day 7, well into the chronic destructive phase, no significant change occurred in the AI of the animals (AI=5.8 ± 0.4 for SCW-treated animals vs. AI=4.3 ± 0.3 for SCW+DR-treated animals at day 7). A single injection of DR 1 day before SCW administration did not diminish the acute or chronic phases of the arthritis (AI=12.4 ± 1.3 for SCW animals vs. AI=9.5 ± 0.5 for SCW+ DR animals at day 7; data not shown).

3. Inhibition of leukocytosis by TGF β1 and DR
The marked reduction in inflammatory cell infiltrate
prompted sequential analysis of the effects of TGF-$\beta$ and $\text{DR}$ on circulating hematopoietic cells. On day 3, the number of circulating WBCs was elevated for wall SCW-treated animals, regardless of any TGF-$\beta$+DR treatment: $13.4 \pm 1.1 \times 10^3/\text{mm}^3$ (n=5) for all SCW-injected animals with or without daily TGF-$\beta$+DR treatment as described in Fig. 1. The data are expressed as mean number of WBCs $\times 10^3/\text{mm}^3$ blood $\pm$ SEM (n=5) for each group.

Fig. 5. Decrease in the number of circulating WBCs after TGF-$\beta$1+DR treatment on day 24. Total WBC count for control and streptococcal cell wall (SCW)-injected animals were determined on day 24 with or without daily TGF-$\beta$1+DR treatment as described in Fig. 1. The data are expressed as mean number of WBCs $\times 10^3/\text{mm}^3$ blood $\pm$ SEM (n=5) for each group.

Discussion

Oriental medicines, which have been developed over some 3,000 years and are known to have low toxicity, may offer advantages for the longer term use over the synthetic drug agent medication. Although the acting preventive mechanism of these oriental medicines remains to be explained, this initial study of $\text{DR}$ does show that $\text{DR}$ is effective for gynecological diseases such as osteoporosis. Plants used in folk medicine have been accepted as one of the main sources of drug discovery and development. In Korea, there is a rich treasury of ethnobotanical knowledge. During our field studies, we have coincided the $\text{DR}$ claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. A literature survey about $\text{DR}$ revealed that there is little scientific evidence of its usefulness in the treatment of RA and osteoporosis.

Previously, it was shown that the inhibition effect of bone resorption and collagenolysis was caused by in vitro PGE2-stimulated IL-1$\beta$ production and cAMP-PKA signaling pathway to regulate IL-1$\beta$. The $\text{DR}$ showed the inhibitory effects against the increase of the PGE2-stimulation. The preventive effects of $\text{DR}$ on the progress of bone loss induced by ovariectomy in rats were investigated for a period of 6 weeks. The bone mineral density of tibia in ovariectomized rats decreased by 22% that those in sham-operated rats, with the decrease completely inhibited by the administration of the $\text{DR}$ or 17-beta-estradiol. The administration of the $\text{DR}$ and 17beta-estradiol to ovariectomized rats preserved the fine particle surface of the trabecular bone. The $\text{DR}$ extract strongly inhibited PGE2- and LPS-stimulated IL-1$\beta$ production. Pretreatment of the $\text{DR}$ after 1 h and 24 h of treatment also suppressed the IL-1$\beta$ production. The $\text{DR}$ extract strongly inhibited the PGE2-stimulated IL-1$\beta$ transcription. $\text{DR}$ was as effective as 17-beta-estradiol in preventing the development of bone loss induced by ovariectomy in rat and that the $\text{DR}$ is effective for anti-bone resorptive action in bone cells.

In this study, the anti-arthritic action of $\text{DR}$ in the
SCW-induced model of mice and rats were characterized. DR intraperitoneal (i.p.) treatment in itself does not affect the physiological immunological responses in the tissues. The anti-arthritis effect of DR was evident in vitro and in vivo. An intraperitoneal route of cytokine delivery was chosen over intravenous injection because the serum component, \( \alpha_2 \)-macroglobulin, is known to effectively bind TGF\( \beta \). Daily intraperitoneal administration of TGF\( \beta \) and DR to SCW-treated animals resulted in a marked suppression of the acute and chronic phases of SCW-induced arthritis.

The decreased inflammatory cell recruitment into the synovium of the TGF\( \beta \) and DR -treated animals may be due to an inhibition of the SCW-induced leukocytosis. SCW-treated animals typically manifest an increasing number of circulating leukocytes, which serve as a reservoir of cells for recruitment into the joints and other sites of chronic inflammation\(^{22} \). Treatment of TGF\( \beta \) and DR was found to suppress the increase in the number of circulating leukocytes, suggesting that the inhibition of leukocytosis may be important in preventing the arthritic condition. This effect was noted during acute phase, but was observed consistently in the chronic phase and was dependent on the amount of TGF\( \beta \) and DR administered. The inhibition of SCW-induced leukocytosis may be due to a decrease in the proliferation of hematopoietic precursor cells in the bone marrow. Administration of TGF\( \beta \) to mice via the femoral artery has recently been demonstrated to cause the partial inhibition of bone marrow proliferation\(^{23} \). Several in vitro studies support this observation\(^{24,25} \). Thus, the limited recruitment of inflammatory cells into the joint may be due, in part, to a lower number of circulating WBCs.

Also, TGF\( \beta \) has been identified as a potent monocyte chemotactic factor\(^{26} \). Exposure of circulating human monocytes to TGF\( \beta \) and DR effectively downregulates TGF\( \beta \) receptor expression, indicating that a diminished pool of circulating WBC and a decreased chemotactic response to TGF\( \beta \) and DR might effectively restrict the synovial inflammatory response dependent on the cell recruitment. TGF\( \beta \) has also known to inhibit neutrophil adhesion to endothelial cells, the event preceding cell migration into the tissue\(^{27} \). Systematic administration of TGF\( \beta \) and DR may decrease blood cell adhesion to the endothelium, thus also limit the inflammatory cell recruitment into the joint. Also, recently it was shown that the TGF\( \beta \) decreases IL-1 receptor expression\(^{28} \) and the production of superoxide radical both in vitro\(^{29} \) and in vivo\(^{30} \).

TGF\( \beta \) inhibits the IL-1\( \beta \)-induced chondrocyte protease activity and the cartilage proteoglycan degradation\(^{31-33} \). Furthermore, TGF\( \beta \) inhibits the formation of osteoclast-like cells in long term human bone marrow cultures\(^{34} \) and inhibits bone resorption\(^{35} \). TGF\( \beta \) and DR was shown in this study to effectively inhibit the development of an induced arthritic condition in rats, likely via its immunoregulatory effects and its inhibition of connective tissue degradation\(^{36} \).

Therefore, the need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study. This result also suggested that the DR extracts is effective for anti-arthritic effects.

References

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