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

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Drynariae Rhizoma (*DR*), an herbal medicine known to clean blood and improve its circulation, frequently appears as the main ingredient in the prescriptions for bone injuries. Currently, it is unclear how contributes pharmacologically to the reformation of bone. Therefore, we have done this study. Systematic administrations of TGFβ1 and water extract of *DR* diminished the polyarthritis development in rats. TGFβ administration (0.1-2 μg/animal) and *DR* (10-100 μg/animal), initiated 1 day before an arthritogenic dose of streptococcal cell wall fragments, virtually eliminated the joint swelling and distortion observed during the acute phase and the chronic phase of the disease. The TGFβ and *DR* synergistically suppressed the arthritis when the administration was begun after the acute phase of arthritis. Also, the synergistic activity between TGFβ and *DR* was confirmed in their suppression of arthritis in rats. Consistent with the inhibition of inflammatory cell recruitment into the synovium, TGFβ1 and *DR* reversed the leukocytosis associated with the chronic phase of the arthritis.

Key Words: *Drynariae Rhizoma*, arthritis, leukocytosis, streptococcal cell wall (SCW) transforming growth factor (TGF)

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[1,2]. 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 tonifing the kidney, invigorating the blood and stopping the bleeding in the Korean and Chinese medicinal literature[3]. Since a large decrease in bone mass occurs in the postmenopausal state, women are vulnerable to the type of osteoporosis known as postmenopausal osteoporosis[4]. *DR*, named Gol-se-bo in Korea is the root of *Drynariae Rhizoma* (Oliv.), an herbaceous perennial plant belonging to the *Drynaria* Family[3]. *DR*

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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 raging 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[8]. 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-1β mRNA transcription and so, LPS-stimulated inflammatory IL-1β production[8]. 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[9,10].

The potent immunosuppressive effects of transforming growth factor β (TGFβ)[11], suggest that it may be valuable in the treatment of disease states characterized by aberrant functions of the immune system. TGFβ 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 immunoglo-bulins[12]. Since many of the immune cell functions influenced by TGFβ are involved in the sequence of events leading to connective tissue destruction in arthritic lesions, TGFβ may be effective for suppressing the pathogenesis for the chronic inflammatory disease. To examine the TGF β1 effects on arthritis animals, TGFβ1 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 TGFβ1-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 TGFβ.

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 Pharmacopoeia[15]. 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℃ 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℃. 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℃, 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 Biomeda (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 described[13]. 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 TGFβ 1/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 described[14,18]. After 68 h of culture, the cells were pulsed for 4 h with 0.5 μCi/well of [^{3}H]thymidine ([^{3}H]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 $\pm$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 TGFβ 1 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 TGFβ /rat was administered daily during development of the arthritic response, the rats displayed a very blunted acute inflammatory phase and virtually no chronic inflammatory phase. Even a dose as low as 0.2 μg TGF β1/rat daily resulted in a decrease in the severity of the joint inflammation during the acute and chronic phases and a delay in the onset of the chronic inflammation (Fig. 1A).

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 TGF β and DR

Because of the profound effect of TGFβ and *DR* on

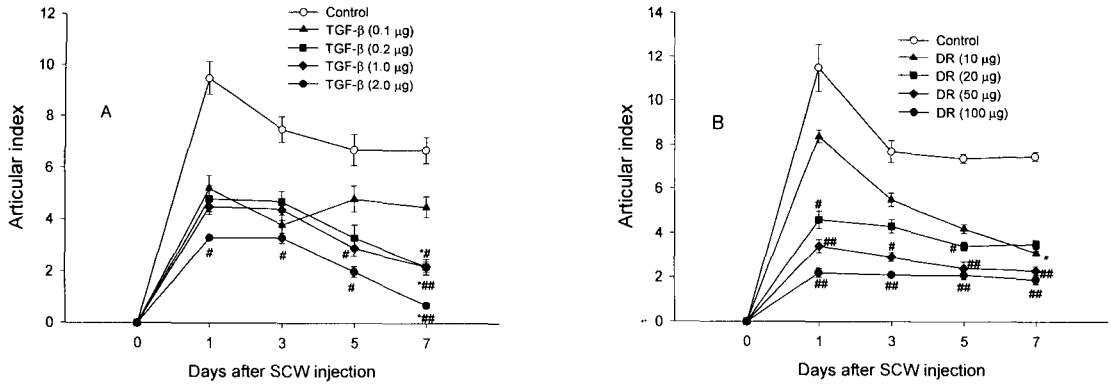

Fig. 1 Modulation of SCW-induced arthritis by various doses of TGFβ,1 (A) and DR (B). Animals were injected with streptococcal cell wall (SCW) on day 0. Control animals received no additional treatment; others were treated with TGF β1 (i.p.) daily at 0.1, 0.2, 1.0 and 2 μg/kg on the beginning 1 day before SCW injection. And, the another animals were treated with *DR* (i.p.) daily at 0.1, 0.2, 1.0 and 2 μg/kg on the beginning day before SCW injection. Each point represents the mean joint score for each group of animals (n=5). Data for all the other groups of control animals (PBS, vehicle, and TGFβ1-injected) are not shown; all the other control animals had mean joint scores of zero throughout the experiment. The experiment was repeated with some modifications three times with similar results. * $p<0.05$ compared with each control (1 day). #, ## $p<0.05$ and $p<0.01$ compared with the same day.

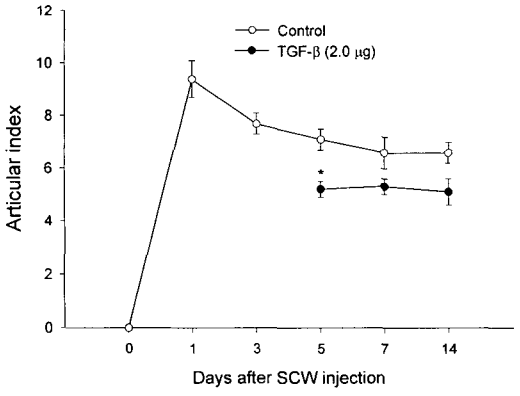

Fig. 2. The effect of TGF β1 treatment on the chronic phase of the arthritis. Lewis rats were injected with the streptococcal cell wall (SCW) on day 0 and their articular index determined daily thereafter. On day 5, half of the animals were begun on a daily dosing regimen of 2 μg TGF β1/rat (i.p.). Joint scores for all the other control animals were the same as indicated in Fig. 1. * $p<0.05$ compared with the same day.

the delevelopment of arthritic lesions when administration was begun before the onset of detectable inflammation, it was next investigated whether TGFβ 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 TGF β1 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

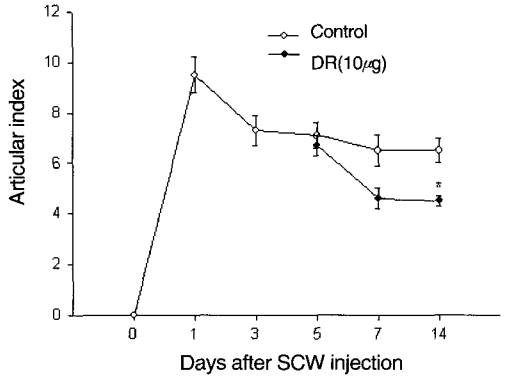

Fig. 3. The effect of *DR* treatment on the chronic phase of the arthritis. Lewis rats were injected with the streptococcal cell wall (SCW) on day 0 and their articular index determined daily thereafter. On day 5, half of the animals were begun on a daily dosing regimen of 10 μg *DR*/rat (i.p.). Joint scores for all the other control animals were the same as indicated in Fig. 1B. *, $p<0.05$ compared with the same day.

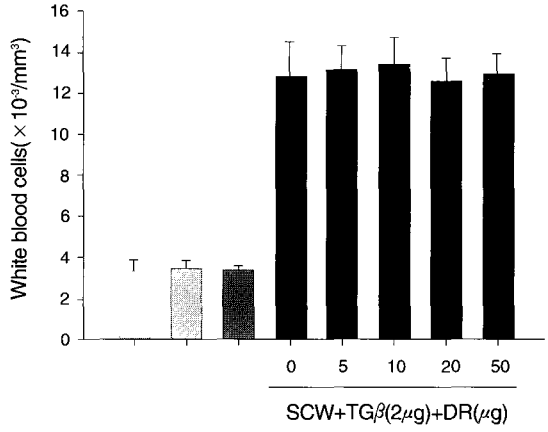

Fig. 4. Changes in number of circulating WBCs after TGF β 1+*DR* treatment on day 3. Total WBC count for control and streptococcal cell wall (SCW)-injected animals were determined on day 3. Some of the animals were treated with TGF β+*DR* daily as described in Fig. 1A. The data is expressed as mean number of WBCs × 10^{-3}/mm³ blood SEM (n=5) for each group.

injections of TGFβ1 were begun on day 7, well into the chronic destructive phase, no significant change occured in the AI of the animals (AI=6.3±1.4 for SCW-treated animals vs. AI=5.3±1.0 for SCW+TGFβ 1-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=10.5±1.4 for SCW animals vs. AI=8.8± 0.6 for SCW+TGFβ1 animals; day[14]) (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[14]) (data not shown).

3. Inhibition of leukocytosis by TGF β1 and *DR*

The marked reduction in inflammatory cell infiltrate

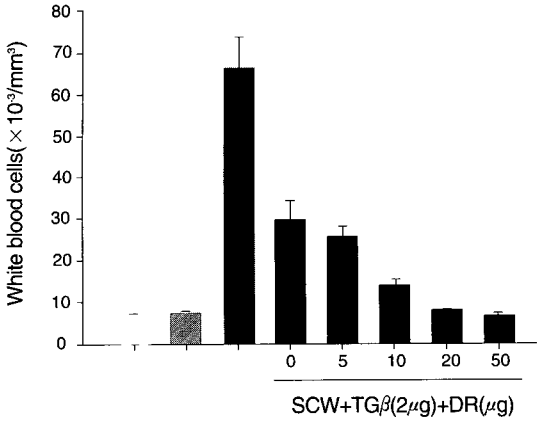

Fig. 5. Decrease in the number of circulating WBCs after TGFβ1+*DR* treatment on day 24. Total WBC count for control and streptococcal cell wall (SCW)-injected animals were determined on day 24 with or without daily TGFβ1+*DR* treatment as described in Fig. 1. The data are expressed as mean number of WBCs x 10^{-3}/mm^3 blood SEM (n=5) for each group.

prompted sequential analysis of the effects of TGF-β and *DR* on circulating hematopoietic cells. On day 3, the number of circulating WBCs was elevated for wall SCW-treated animals, regardless of any TGFβ1+ *DR* treatment: $13.4 \pm 1.1 \times 10^{-3}$/mm^3 (n=5) for all SCW-injected animals with or without TGFβ1+ *DR* vs. $3.4 \pm 0.2 \times 10^{-3}$/mm^3 (n=5) ($p<0.05$) for the controls (Fig. 4). However, by day 24, daily systemic administration of TGFβ1+ *DR* had significantly suppressed the elevated WBC count associated with the inflammation in a dose-dependent manner (Fig. 5). Hematocrits measured on day 14 were not suppressed as compared with those at day 1 (data not shown).

Discussion

Oriental medicines, which have been developed over some 3,000 years[19] 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 *DR* does show that *DR* is effective for gynecological diseases[1,2] 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[20]. During our field studies, we have coincided the *DR* claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. A literature survey about *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-1β production and cAMP-PKA signaling pathway to regulate IL-1β. The *DR* showed the inhibitory effects against the increase of the PGE2-stimulation[8]. The preventive effects of *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 *DR* or 17-beta-estradiol. The administration of the *DR* and 17beta-estradiol to ovariectomized rats preserved the fine particle surface of the trabecular bone. The *DR* extract strongly inhibited PGE2- and LPS-stimulated IL-1β production. Pretreatment of the *DR* after 1 h and 24 h of treatment also suppressed the IL-1β production. The *DR* extract strongly inhibited the PGE2-stimulated IL-1β transcription. *DR* was as effective as 17-beta-estradiol in preventing the development of bone loss induced by ovariectomy in rat and that the *DR* is effective for anti-bone resorptive action in bone cells[8].

In this study, the anti-arthritic action of *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, α2-macroglobulin, is known to effectively bind TGFβ[21]. Daily intraperitoneal administration of TGFβ 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 TGFβ 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 β 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 TGFβ 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 TGFβ 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, TGFβ has been identified as a potent monocyte chemotactic factor[26]. Exposure of circulating human monocytes to TGFβ and *DR* effectively downregulates TGFβ receptor expression, indicating that a diminished pool of circulating WBC and a decreased chemotactic response to TGFβ and *DR* might effectively restrict the synovial inflammatory response dependent on the cell recruitment. TGFβ has also known to inhibit neutrophil adhesion to endothelial cells, the event preceding cell migration into the tissue[27]. Systematic administration of TGFβ 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 TGFβ decreases IL-1 receptor expression[28] and the production of superoxide radical both *in vitro*[29] and in vivo[30].

TGFβ inhibits the IL-1β-induced chondrocyte protease activity and the cartilage proteoglycan degradation[31-33]. Furthermore, TGFβ inhibits the formation of osteoclast-like cells in long term human bone marrow cultures[34] and inhibits bone resorption[35]. TGFβ 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.

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