Effects of the Butanol Extract of Head of Panax Ginseng on Type II Collagen-induced Arthritis in DBA/1J Mice

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Abstract – In order to evaluate the improvement effects of head of Panax ginseng on chronic arthritis, we have investigated the activity of butanol fraction (BuOH fraction) in vitro and in vivo system. BuOH fraction showed significant inhibition on the elastase activity. Anti-arthritic activity of BuOH fraction was also examined on type II collagen-induced arthritis in DBA/1J mice. Mice were immunized with injection of type II collagen emulsified in Freund’s complete adjuvant, followed by a booster injection 21 days later. BuOH fraction (BHPG) was administered at an oral dose of 500mg/kg for 2 weeks from the 1st day boost. The hind paw edema was significantly decreased in the group of treatment with BuOH fraction compared to control. In collagen-induced DBA/1J mice, BuOH fraction did not affected the collagen antibody titer but significantly inhibited the tumor necrosis factor-alpha (TNF-α) activity. These results were confirmed with histological evaluation of joint tissues. This study may raise the possibility that the usage of BuOH fraction of head of Panax ginseng as alternative medicine for the relief and prevention of rheumatoid arthritis symptoms.

Keywords □ Head of Panax ginseng, inflammation, arthritis, elastase, TNF-α

INTRODUCTION

Rheumatoid arthritis (RA), an autoimmune diseases of unknown origin, is associated with inflammation of joints and a variety of systemic problems (Grassi et al., 1998). Macrophages play an important role in inflammatory disease, by production of the cytokines, interleukin-1 beta (IL-1β) and tumor necrosis factor (TNF-α), other inflammatory mediators, nitric oxide (NO) and prostangladins. Production of these mediators from macrophages has been found in many inflammatory tissues and overproduction of the inflammatory mediators involves many diseases, such as rheumatoid arthritis, artherosclerosis, chronic hepatitis and pulmonary fibrosis. The presence of inflammatory lymphokines such as IL-1, IL-4, TNF-α as well as TGF-β, can be detected immunohistochemically in the synovium. Many disease-modifying drugs, such as methotrexate, appear to suppress the introduction of TNF-α and IL-1 (Bondeson, 1997; Madhok et al., 1993).

The most widely used model for rheumatoid arthritis is the collagen-induced arthritis (CIA) in mice. Intradermal immunization of certain strains of mice or rats with heterologous type II collagen (CII) emulsified in and adjuvant produces a form a rheumatoid arthritis that resembles the human form of the disease. CII is a major protein constituent of joint cartilage and the immunization provokes an autoimmune response that attacks the joints. The two arms of adaptive immunity, T and B cells play a central role in the pathogenesis of CIA but their relative importance in both priming of immune activation and joint destruction are still unclear. The major role of B cells is production of arthritogenic anti-CII antibodies, which is clearly shown by the fact antibodies reactive with CII can bind to cartilage and induce arthritis (Terato et al., 1996). The role of T cells in CIA is more complex and can be divided into two main pathways which synergize in the development of arthritis. Firstly, T cells provide help to B cells for the production of arthritogenic anti-CII antibodies (Corthay et al., 1999). Secondly, T cells themselves are believed to play a role in joint inflammation through activation of other cells, e.g. synovial macrophages. Transfer of T cells can induce synovitis but not clinically evident arthritis and blockage of T cells or T cell function ameliorate development of arthritis (Holmdahl et al., 2002). Kallias and co-workers have found that transgenic mice over-expressing human TNF-α rapidly and spontaneously develop a severe ankylosing arthritis that is independent of the action of T or B
cells (Probert et al., 1993; Hom et al., 1998). This observation demonstrated that increased TNF-α expression causes arthritis.

The root of Panax ginseng is a traditional medicine used for the treatment of various diseases in Asian countries, such as Korea, Japan, and China, where it is called ‘Insam’ or ‘Ginseng’. Head of Panax ginseng indicates its growth number of years and it has been widely used for supplying energy to weaklings and emesis.

We performed a pharmacological investigation on the antiarthritic activity of the BuOH fraction using in vitro test and CIA animal models. This evidence suggests that BuOH fraction regulates the production of inflammatory mediators. However, the biological and pharmacological effects of BuOH fraction on inflammatory mediator production have not been yet elucidated.

MATERIALS AND METHODS

Materials

Human leukocyte elastase [E.C. 3.4.21.37], type II collagen and alkaline phosphatase substrate (p-nitrophenyl phosphate disodium) were obtained from Sigma (St Louis, MO). Chromogenic substrate, Boc-Ala-Ala-Pro-Ala-p-nitroanilide and alkaline phosphatase-conjugated anti-mouse IgG antibody were purchased from Calbiochem (La Jolla, CA). The enzyme-linked immunosorbent assay (ELISA) kits for murine TNF-α were from R&D Systems (Minneapolis, MN), other reagents and chemicals were of the best grade available.

Animals

Male DBA/1J mice, aged 5-6 weeks, were purchased from Japan SLC Inc. (Shizuoka, Japan) and acclimatized to standard laboratory conditions (25±3°C, 55±5% humidity and 12 h light/dark cycle) for 7 days. All animal work was carried out in a specific pathogen-free barrier zone at Seoul National University Hospital in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals.

Plant materials and preparation of the BuOH fraction

Head of Panax ginseng (HPG) was purchased at keung dong herbal market in Seoul, Korea. HPG (10 kg) was extracted with methanol (MeOH) 4 times under reflux for 5 h. The MeOH solutions were combined, filtrated and concentrated to yield a dry MeOH extract (2616 g). The MeOH extract was suspended in distilled water and fractionated with hexane, ethyl acetate (EtOAc), and butanol (BuOH) to give a hexane soluble fraction (120 g), an EtOAc soluble fraction (220 g), and a BuOH soluble fraction (923 g), respectively.

Effect of BuOH fraction on the inhibition of elastase activity

This assay was essentially performed according to a previous report with some modifications (Volpi, 1996). Briefly, each sample (40, 100, 200 μg/mL) was dissolved in 165 μl of 50 mM Tris-HCl with 0.05 M NaCl (pH 8.0). Human leukocyte elastase (15 μl, 1 mU/μl) was added in the same buffer and the mixture was incubated for 3 min at 37°C. One hundred microliters of 0.5 mM of the chromogenic substrate (Boc-Ala-Ala-Pro-Ala-p-nitroanilide) was added and the mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 100 μl of 0.5 M acetic acid and the absorbance was measured at 405 nm. The percent inhibition of elastase activity was calculated as residual activity versus a control.

Induction of arthritis and treatment of BuOH fraction

Type II collagen was dissolved overnight at 4°C in 0.05 mol/l acetic acid to 2 mg/ml. This solution was then emulsified in an equal volume of complete Freund’s adjuvant in an ice-cold water bath. Arthritis was induced by the intradermal injection of 0.1 ml of this emulsion into the base of the tail. Mice were boosted using the same schedule 21 days later.

BuOH fraction was administered at an oral dose of 500 mg/kg for 2 weeks from the 1st day boost. The arthritic control group received an equivalent volume of physiological saline based on body mass according to the same schedule.

Measurement of hind paw edema and preparation of serum

Edema in the hind paw was measured before the initial injection of type II collagen and after the booster injection, using a digital gauge. The mean thickness of the right and left hind paws was recorded as paw thickness over a period of 49 days.

Blood was collected by heart puncture. After clotting at room temperature, the blood was kept overnight at 4°C and the serum was collected by centrifuging at 2,000 x g for 15 min. All samples were stored at -80°C until required.

Measurement of anti-type II collagen antibody titer

Anti-type II collagen antibody titer was determined as described previously (Omata et al., 2000). In brief, a 96-well ELISA plate was coated with 10 μg/ml bovine type II collagen in 50 mM sodium carbonate buffer, pH 9.8 containing 5 mM MgCl₂ (100 μl/well), incubated at 37°C for 1 h and then left
overnight at 4°C. After washing with PBS-Tween 20 three times, the plates were blocked with PBS containing 1% BSA at 25°C for 1 h. Sera diluted 1:10,000 in PBS-Tween 20 were added to 96-well plates coated with type II collagen and incubated overnight at 4°C. After washing with PBS-Tween three times, alkaline phosphatase-conjugated anti-mouse IgG antibody diluted at 1:10,000 in PBS-Tween 20 was added to each well and the plate was incubated at 37°C for 1 h. The plate was then rewashed and then the substrate (p-nitrophenyl phosphate disodium, 10 mg/mL in 50 mM sodium carbonate buffer, pH 9.8, containing 5 mM MgCl₂) was added. The absorbance was measured at 405 nm with an E-max microplate reader (Molecular Device, Sunnyvale, CA)

Effects on cytokines in serum

Cytokine levels in serum were determined using enzyme-linked immunosorbent assay (ELISA) kits for murine TNF-α (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions at room temperature.

Histological evaluation

Exirpated joint tissues were fixed for 48h with 4% formalin, decalcified in 10% formic acid-formalin solution, dehydrated in different mixtures of ethyl alcohol-water (50, 80, 95%) and finally in absolute alcohol, cleared in xylene and embedded in paraffin. Sections (4-5 μm thick) were prepared and then stained with hematoxylin-eosin dye for microscopic observation (100×).

Statistical evaluations

Data are expressed as means±S.D. To analyze the data statistically, we performed one-way analysis of variance (ANOVA) for repeated measurements of the same variable, and used Duncan’s multiple range t-test to determine which means were significantly different from that of the control. We considered differences significant at p<0.01 and p<0.05.

RESULTS AND DISCUSSION

Effect of BuOH fraction on the inhibition of elastase activity

BuOH fraction group significantly inhibited elastase activity which infiltrates of polymorphonuclear leukocytes into the synovial cavity at 40, 100, 200 μg/ml as shown in Fig. 1.

Neutrophil elastase, a granule serine protease, is a member of the proteinase family, which hydrolytically degrade connective components, such as elastin, proteoglycan, fibronectin and collagen types I, II, III and IV (Havemann and Gramse, 1984). Because elastase can readily degrade matrix proteins, the inhibitors are likely the therapeutic advantage in these pathogenic conditions of arthritis (Fujie et al., 1999).

Induction of arthritis and measurement of hind paw

After a booster injection of type II collagen was administered on day 21, hind paw thickness peaked on day 32 and then gradually subsided Fig. 2. In collagen-induced DBA/1J mice, BuOH fraction group showed significant change of hind paw thickness compared with arthritis control (Fig. 3).

Measurement of anti-type II collagen antibody titer

On day 49, the levels of type II collagen antibodies were measured, and were found to be higher in arthritic control mice
Type II collagen in cartilage is normally exposed to the immune system, and since CII reactive B cells and T cells are of major importance in the pathogenesis of CIA, a fundamental question for understanding CIA, and probably RA as well, is how the immune system is made tolerant to cartilage proteins. BuOH fraction group showed no significant influence of anti-type II collagen antibody.

**Effects of BuOH fraction on the production of cytokines in serum**

In DBA/1J mice, BuOH fraction suppressed TNF-α production in serum as shown in Table II. TNF-α production in serum of arthritis control group and BuOH fraction were 50.3±16.0 and 37.4±5.3 pg/ml, respectively.

Although the etiology of rheumatoid arthritis remains unknown, the excessive production of proinflammatory cytokines, including TNF-α, IL-1 and IL-6, has been implicated as a major factor in the pathogenesis of the diseases. Moreover, both TNF-α and IL-1 have been detected in the joints of rheumatoid arthritis patients (Chu et al., 1991; Chu et al., 1992;) and are known to possess properties, which are consistent with a pathogenic role. TNF-α and IL-1 seem to function synergistically to induce the synthesis and secretion of IL-6 as effectors. It has been well demonstrated that TNF-α, IL-1 and IL-6 are highly expressed at sites of diseases activity in collagen-induced arthritis (Marinova-Mutafchieva et al., 1997). Thus, the regulation of these cytokines in synoviocytes or the synovial environment may be important in the pathogenesis and therapy of rheumatoid arthritis (Adrend and Dayer, 1990).

**Histological evaluation**

Polyester wax sections (width, 10 mm; length, 10 mm; thickness, 6 μm) of the tissue samples were prepared histochromically, and four sections were prepared and stained. The light micrographs of these stained tissue samples are shown in Fig. 4. Figs. 4(A) is section of the negative control group (B) is sections of the arthritic control. In comparison with the negative control group, which had a thin synovium, the arthritic control with arthritis showed cartilage destruction over the articular surface (pannus). 4(C) is sections from cartilage of mice treated with BuOH fraction. In collagen-induced DBA/1J mice, BuOH fraction reduced morphologic and histopathologic changes at a dose of 500 mg/kg. BuOH fraction significantly inhibited the loss of superficial layer and the amount of cartilage erosion.
CONCLUSION

As stated above, we found that BuOH fraction was associated with significantly decreased serum levels of TNF-α, compared with the arthritic control. It also significantly decreased hind paw edema, which can be related to the reduction of these cytokines and the inhibition of the elastase activity. Therefore Anti-inflammation and anti-reumatoid arthritis of BuOH fraction were confirmed by inhibition of TNF-α and HLE activities. Our study demonstrates the possibility that BuOH fraction may be therapeutically useful in rheumatoid arthritis with accompanying chronic inflammation.

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REFERENCES


