원저

Inhibitory Effects of Deer Antler Herbal-Acupuncture Solution on Cathepsin S and L Activity in RA Model Mice

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Abstract

Objective : Dear antler (*Cervus korean* TEMMINCK var. *mantchuricus Swinhoe)* used for traditional immunosuppressive and immuno-activating action. The effect of deer antler herbal-acupuncture(DAH) solution, prepared by water extract method, on cathepsin activities in bone tissues (cartilage and synovial) cells from mouse rheumatoid arthritis (RA) model was studied. The cysteine endoprotease cathepsin mediates degradation of the MHC class II invariant chain (Ii) in human and mouse antigen-presenting cells. The studies described here examine the functional significance of cathepsin inhibition on autoantigen presentation and organ-specific autoimmune diseases in a murine model for RA.

Methods : An animal model for RA in BALB/c mice thymectomized 3 days after birth (3d-Tx) was constructed. All 3d-Tx BALB/c mice developed autoimmune lesions in the bone tissue cells, starting at 3 weeks of age, and the disease mediated by CD4+ T cells was chronic and progressive. Significant inhibitory effects of DAH solution on cathepsin S and L were observed in each organ in a dose-dependent manner. Moreover, we confirmed that cathepsin S and L activity in each organ were clearly inhibited by DAH solution. When we examined the inhibitory effects of DAH solution against autoantigen-specific T cell responses in vitro, in regional lymph node cells, but not in spleens, from model mice, a significant inhibitory effect of DAH solution was observed in a dose-dependent manner. DAH solution do not block T cell proliferation to Con A, indicated that the dose of DAH solution 10 to 20 ㎍/㎖ was sufficient to inactivate the autoantigen-specific T cell responses in vitro. In vivo therapeutic effects of DAH solution were examined in a murine model for RA, autoantigen-specific (C-II-specific) T cell response were significantly inhibited in LNCs from DAH solution-treated mice.

Results : *I*nhibition of cathepsin S and L in vivo alters autoantigen presentation and development of organ-specific autoimmunity in RA model.

Conclusion : These data identify selective inhibition of cysteine protease cathepsin S and L as a potential therapeutic strategy for autoimmune disease process such RA. Thus, DAH solution will served as a potent anti-inflammatory and anti-arthritic agents for treatment of human RA.

Key words : cathepsin S, cathepsin L, invariant chain, rheumatoid arthritis, deer antler herbal-acupuncture(DAH)

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Ⅰ. Introduction

Unossified horn or pilose antler cut from deer which belong to the Cervidae is generally termed "Nokyong" in korean. Nokyong is one of the most famous Korean traditional medicines and has been considered to possess sexual-reinforcing and anti-aging efficacy. Thus, deer antler herbal-acupuncture(DAH) has been used to invigorate the kidney-yang (腎陽), to replenish vital essence and blood and strengthen muscle and bones in a traditional Korean medicine. Water extract of DAH, prepared from the pilose antler of Cervus korean TEMMINCK var. mantchuricus Swinhoe (Nokyong), a traditional immunosuppressive and immuno-activating Korean herbal-acupuncture, has sometimes been compounded in recent Korean commercial restorative, although little is yet known about the pharmacological effects or active ingredients. Extract from DAH by water boiling methods, has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis (RA) and satisfactory results are obtained[1-2]. However, little is known about the mode of action of this traditional medication on RA.

Normal joint function depends upon the structural integrity of the constituent cartilage and bone components, which in turn is dependent upon the equilibrium between the processes of tissue synthesis and degradation during the remodeling of cartilage and bone. It is generally accepted that proteolytic enzymes are involved in the catabolic aspect of normal tissue remodelling[3-4], and that the altered activity of these enzymes is responsible for the cartilage destruction and bone erosion associated with degenerative disorders such as RA. In Korean oriental medicine, RA has been called several different name. For example, there are following several different names in korean : Bi(痺), Yeokjeolpung(歷節風), Baekhoyeokjeolpung(白虎歷節風), Tongpung(痛風), Yupungseupseong arthritis(類風濕性關節炎) and so on[5].

We have screened specific inhibitory effects of DAH solution in vivo as well as in vitro[6-7]. To address this important issue, antigen processing and presentation after specific inhibition of cathepsins by traditional medications were examined in a murine model for RA. In the present study, animal models for RA in BALB/c mice thymectomized 3 days after birth (3d-Tx) was successfully constructed. All 3d-Tx BALB/c mice developed autoimmune lesions in the bone tissue cells, starting at 3 weeks of age, and the disease mediated by CD4+ T cells was chronic and progressive. The role of antigen-presenting cells (APCs) in organ-specific T cell activation in this model has been analyzed. Studies presented here suggest that the inhibition of cathepsin S and L through traditional medication such as DAH solution has important functional consequences in modulating the autoimmune response.

Ⅱ. Materials and Methods

1. Materials

We purchased DAH powder, a water extract of DAH from Gyeongju Oriental Medical Hospital, Dongguk University, Gyeongju-si, Gyeongsangbuk-do, Korea, which is usually used for an i.p injection grade for human. Each powder contained 100 μg of the extract. For i.p. injection into mice, randomly selected powder was ground and suspended in normal

saline at a concentration of 50 μg/10 μl.

All reagents (including enzyme assay substrates) were obtained from Sigma Co or Bachem, Bubendorf, Switzerland, and were of analytical grade where available.

2. Animals

Female BALB/c-strain mice of Genetic Resources center, Korea Research Institute of Bioscience and Biotechnology, (Daejeon, Korea) and were reared in our specific pathogen-free mouse colony, and given food and water ad libitum. They were allowed at least 1 week to adapt to the environment (25±3℃, 55±5% humidity and a 12h light/dark cycle) and were used at 7 weeks of age.

3. Measurement of endogenous cathepsin activities

Enzyme (0.05 ㎖ synovial fluid) was incubated with the appropriate assay medium (total volume 0.3 ㎖) at 37℃ (10-120 min), and the reaction terminated by addition of 0.6 ㎖ of ethanol. The fluorescence of the liberated aminoacyl 7-amino-4-methylcoumarin (AMC) was measured by reference to a tetraphenylbutadiene fluorescence standard block (λex 380 nm, λem 440 nm). Assay blanks were run in which the enzyme was added to the medium immediately before ethanol addition. Assay conditions were modified for samples with high enzyme activity such that the extent of substrate utilization never exceeded 15%. Stock substrate solutions (2.5 mmol/l) were prepared in 10% ethanol.

Bone tissue cells, regional lymph nodes, and spleens from 3d-Tx BALB/c RA model, non-Tx BALB/c, and control C57BL/6 mice were used to assay for cathepsin B, L, and S activity. Lysosomes were isolated for the assay by gentle homogenization of samples using a Teflon homogenizer (Microtec Co. Ltd., Funabashi, Japan) pestle in 0.25 M cold sucrose. The suspension was centrifuged at 3,500 g for 10 minutes at 4℃. The supernatant was centrifuged at 25,000 g for 20 minutes at 4℃. The resulting pellet was resuspended with 50 mM acetate buffer (pH 5.0). The suspension fluid was frozen and thawed three times to disrupt lysosomal membranes. After three cycles of freezing and thawing, the fluid was centrifuged and the supernatant was used as a mitochondria and lysosome fraction. Cathepsin activities were assayed with Z-Arg-Arg-methyl coumarylamide (Peptron Co., Daejeon, Korea) as substrate at pH 5.0 for cathepsin B, with Z-Phe-Arg-methyl coumarylamide for cathepsin L, and using the method described[8] for cathepsin S. Cathepsin B or cathepsin L: 50 mmol/l CH_3COOH/CH_3COONa buffer pH 5.5, 2 mmol/l DTT, 0.25 mmol/l CBZ-Phe-Arg-AMC (cathepsin B+L) or 0.25 mmol/l CBZ-Arg-Arg-AMC (cathepsin B only); cathepsin H: 50 mmol/l KH_2PO_4/K_2HPO_4 buffer pH 6.0, 1 mmol/l DTT, 0.5 mmol/l puromycin, 0.25 mmol/l Arg-AMC were also used.

Assay of cathepsin D activity was based on the spectrophotometric procedure of Pennington[9]: 50 mmol/l CH_3COOH/CH_3COONa buffer pH 3.5, 1 mmol/l DTT, 3 mg/㎖ acid-denatured hemoglobin substrate (total assay volume 0.5 ㎖). The reaction was terminated by addition of 0.5 ㎖ 10% PCA, the samples centrifuged at 2000g for 10 min, and the absorbency of acid soluble peptides determined at 280 nm. Assay blanks were run as above.

The reaction was initiated by addition of substrate (10 μM final concentration) after preincubation with the test compound for 3

minutes at 37°C. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured in a fluorescence spectrophotometer (Shimazu Co., Tokyo, Japan). Emission at 460 nm was measured with excitation at 370 nm.

4. In vivo treatment with DAH solution

We examined the in vivo therapeutic effects of DAH solution in a murine model for RA. DAH solution dissolved in PBS, was administered intraperitoneally into model mice at doses of 50 ㎍ mg/mouse/day from 4 weeks to 7 weeks (n = 11 for each), and then analyzed at 8 weeks, compared with untreated RA model mice (n = 7).

5. Histology

All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. The sections were stained with hematoxylin and eosin. Histological grading of the inflammatory lesions was done according to the method proposed by White and Casarett[10]. Three independent pathologists scored these slides in a blinded manner.

6. Proliferation assay

Single-cell suspensions of spleen cells or regional lymph node cells (LNCs) from 3d-Tx, non-Tx BALB/c, and C57BL/6 mice were cultured in 96-well flat-bottom microtiter plates (Nalge Nunc Intl. Co., Rochester, New York, USA) in RPMI-1640 containing 10% FCS, penicillin/streptomycin, and ß-mercaptoethanol. For proliferation assay, a total of 5 x 106 cells per well were cultured for 72 hours under stimulation of type-II collagenn (C-II, 10 ㎍/㎖), ovalbumin (OVA) (10 ㎍/㎖), and concanabalin A (Con A) (5 ㎍/㎖), and pulsed with 1 ㎕Ci/well of [3H]thymidine (NEN Life Science Products Inc., Boston, Massachusetts, USA) during the final 20 hours of the culture. [3H]thymidine incorporation was evaluated using an automated ß liquid scintillation counter. T cell purification was done using CD4 mAb-bounded immunomagnetic beads (Dynal, Oslo, Norway).

7. Primary culture of mouse bone cells

Mouse synovial and cartilage (MSC) cells were prepared as follows: MSCs were dissolved in DMEM/F12 containing 10% FBS. The suspension was passed through sterile 100-㎛ nylon mesh filter and were redigested for 30 minutes by the same digestion procedure, and then the digest suspension was passed through a 100-㎛ nylon mesh filter. Adherent cells, after culture in MEM containing 10% FBS for 24 hours at 37°C, were isolated as bone cells epithelial cells. We confirmed that the cells over 95% were positively stained with anti-keratin polyclonal antibody.

8. Detection of serum autoantibodies against collagen

Serum autoantibody production against collagen was analyzed by immunoblotting and ELISA as described: Blood was collected individually once a week from tail vein, and the serum anti-C-II antibodies titer was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA). Alternatively, blood was obtained from the mice by cardiac puncture under ether anesthesia, after which serum antibody levels to C-II were measured by the ELISA as described[11]. Wells of 96-well microtiter plates (No. 3912, Becton Dickinson, Oxnard, CA) were coated with 100 ㎕ of C-II

at a concentration of 25 μg/mℓ in coating buffer at pH 9.6 for 12 h at 4℃ and washed three times with washing buffer (PBS-0.05% Triton X-100). Wells were blocked with 200 μl of 1% bovine serum albumin 9BSA in PBS for 1h at room temperature, and then washed five times. Aliquots of rat test serum were added to each well (100 μl/well) in duplicate, and incubated for 2h at room temperature. After washing, 100 μl of biotin-conjugated polyclonal goat anti-rat immunoglobulins of IgG, IgM, and IgA (Tago, Burlingame, CA) was dispensed into each well, incubated for 1h, and washed. Streptoavidin-horseradish peroxidase conjugate (Gibco, Life Technologies, Grand Island. NY) was added to each well at a volume of 100 μl/well. After incubation for 45 min, 100 μl of substrate solution (o-phenylenediamine dihydrochloride) (Sigma Co) was added. The reaction was stopped by adding 100 μl of 4 N H2SO4. The optical density (OD) of absorbance at 490 nm was measured with an ELISA reader of SPECTRAmax microplate spectrophotometer (Molecualr Devices, Sunnyvale, CA, USA). The quantity of IgG anti-C-II antibody as anti-collagen titer was expressed as mg/100 mℓ of serum by comparison with standard curves obtained from an affinity-purified rat anti-C-II antibody control. Also, the titer was expressed as the reciprocal dilution at which the OD was 50% of the maxium OD. The subtypes of anti-collagen specific antibody were analyzed by the Rat Typer Sub-isotyping Kit purchased from Bio-Rad Lab. (Richimond, CA, USA).

9. Confocal immunofluorescence analysis

IFN-γ-stimulated and nonstimulated MSF cells were fixed with 1% paraformaldehyde and were incubated with mAb to I-Aq molecule (Pharmingen) and FITC-labeled AFN303-318 peptide (described below). In vitro effects of DAH solution were examined by the preincubation with DAH solution (10 - 20 μg/mℓ) for 6-24 hours. The labeled second antibody was Texas red-conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, Oregon, USA). For microscopy, a Leica TCS-NT laser-scanning microscope (Leica Microsystems Nussloch GmbH, Nussloch, Germany) was used.

10. Protain determination

Synovial fluid protein levels, for calculation of specific protelytic enzyme activity measurements and specific protein carbonyl measurements above, were determined by the method of Lowry et al. using bovine serum albumin as standard.

11. Analytical methods

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

12. Statistical analysis

Results were expressed as means ± SEM. Statistical analysis was performed by Student's t-test with $p<0.05$ for significance.

III. Results

1. Effect of DAH solution on endogenous activities of cathepsin in tissue samples

The activities of both cathepsin S and L were significantly higher in the bone tissue,

lymph nodes, and spleens from model mice than control groups(Fig. 1). No difference in activities of cathepsin B was found in any organ from these mice. Significantly inhibitory effects of DAH solution on cathepsin S and L were observed in each organ in a dose-dependent manner. Moreover, we confirmed that cathepsin S and L activities in each organ were clearly inhibited by DAH solution (Fig. 2). However, the cathepsin S and L activities of non-Tx mice and control mice (C57BL6) when they were administrated with DAH solution were not inhibited (data not shown).

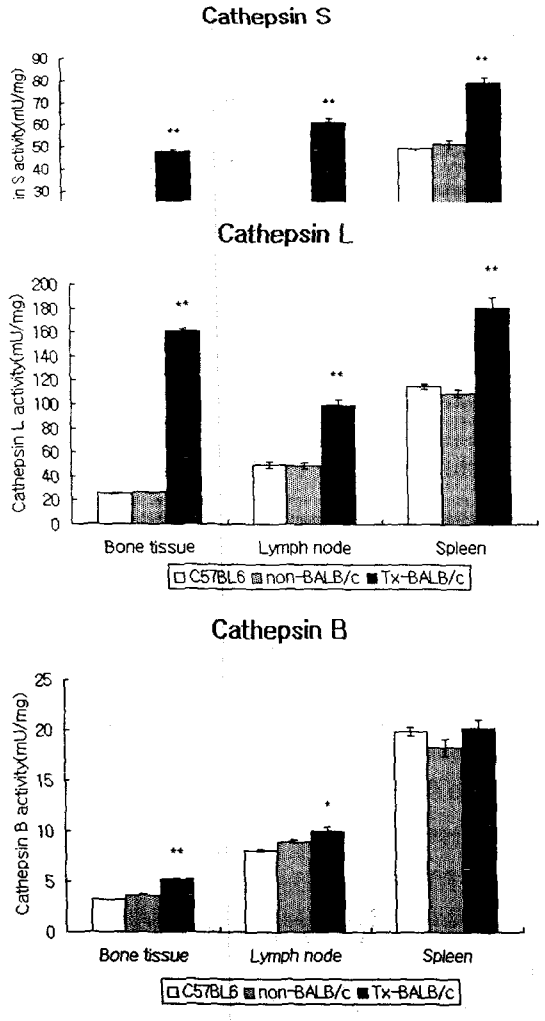


Fig. 1. Endogenous cathepsin activities in tissue samples from RA model mice and control group. The activities of both cathepsin S and L were significantly higher in the bone tissue, regional lymph nodes and spleens from model mice than in those from control group (control group - non-Tx BALB/c, * p < 0.05, ** p < 0.01, Student's t-test).

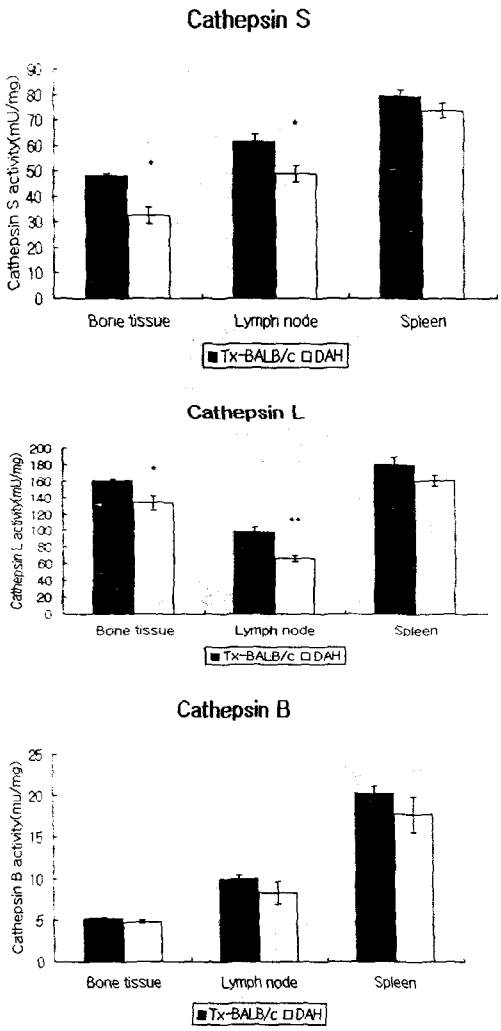
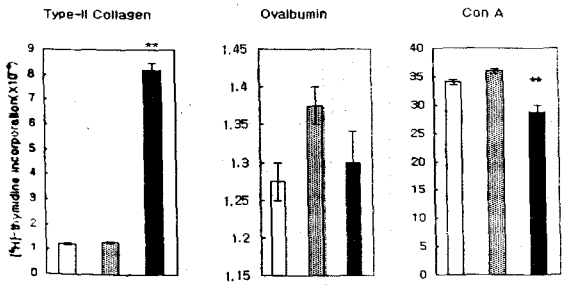


Fig. 2. Effect of DAH solution administration on endogenous cathepsin S, L and B activities in tissue samples from RA model mice. The activities of both cathepsin S and L, which were significantly higher in the bone tissue, regional lymph nodes and spleens from model mice than in those from control group, were significantly decreased (* p < 0.05, ** p < 0.01, Student's t-test).

2. Inhibitory effect of a DAH solution as cathepsin on proliferative T-cell response to autoantigen

We found that the LNCs and spleen cells in RA model mice, but not in non-Tx BALB/c and C57BL/6 control mice, at 8 weeks of age

showed a significant increase in autoantigen-specific(C-II-specific) T cell proliferation (Fig. 3; data of the spleen cells not shown). No significant difference was observed in the proliferative response stimulated with ovalbumin (10 μg/mℓ), however Con A (5 μg/mℓ) decreased the proliferative response with unknown reason among these mice. We examined the inhibitory effects of DAH solution against autoantigen-specific T cell responses in vitro. In regional LNCs, but not in spleens, from model mice, a significantly inhibitory effect of DAH solution was observed in a dose-dependent manner (Fig. 4). Annexin V/propidium iodide (PI) flow cytometric analysis revealed a small proportion of early apoptotic cells (annexin V-positive, PI-negative) and necrotic cells (annexin V-positive, PI-positive), indicating that DAH solution is not toxic and favors T cell survival in the LNCs (not shown). Indeed, the DAH solution at 10 to 20 ug/mℓ concentrations do not block T cell proliferation to Con A (Fig. 5). These findings indicated that the dose of DAH solution 10 to 20 μg/mℓ was sufficient to inactivate the autoantigen-specific T cell



C57BL6(□)non-TxBALB/c(▨) Tx-BALB/c(■)

Fig. 3. Detection of proliferative T cell response of LNCs from RA model mice to organ-specific autoantigen. We found that the LNCs in RA model mice showed a significant increase in type-II collagen-specific T cell proliferation. But no differences was found in ovalbumin, however Con A conferred significant difference(control group - non-Tx BALB/c, ** $p < 0.01$, Student's t-test).

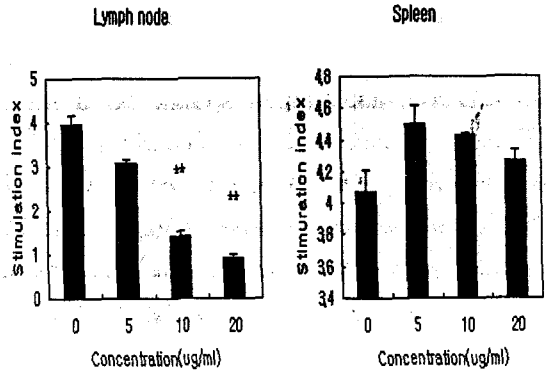


Fig. 4. Preventive effects of proliferative T cell response of LNCs and spleen cells to C-II-specific by DAH solution in a dose-dependent manner. In vitro preventive effects of proliferative T cell responsed in LNCs, but not in spleen cells to C-II-specific by DAH solution in a dose-dependent manner. (** $p < 0.01$, Student's t-test).

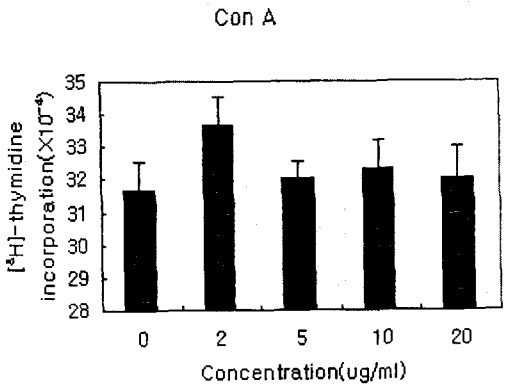


Fig. 5. Preventive effect of proliferative T cell response of LNCs to Con A by DAH solution in a dose-dependent manner. DAH solution at 1 to 20 μg/mℓ concentrations do not block T cell proliferation to Con A in the LNCs.

responses in vitro.

3. Effect of in vivo administration of DAH solution on autoantigen-specific (C-II-specific) T cell proliferation in LNCs, compared with controls

We next examined the in vivo therapeutic effects of DAH solution in a murine model for RA. The activation markers (CD44high, CD45RBlow, Mel-14low) were clearly

downregulated in LNCs gated on CD4 from DAH solution-treated model mice (not shown). In addition, autoantigen-specific (C-II-specific) T cell response was significantly inhibited in LNCs from DAH solution-treated mice (Fig. 6). These results strongly suggest that DAH solution plays an important role in preventing autoantigen-recognizing T-cell proliferation.

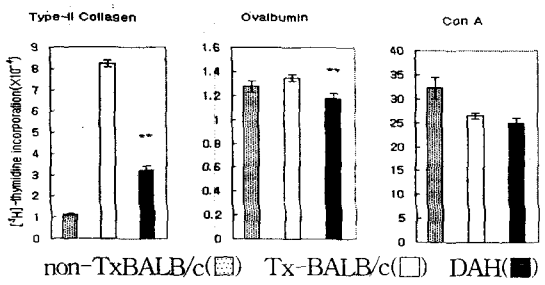


Fig. 6. Effect of in vivo administration of DAH solution on autoantigen-specific T cell proliferation in LNCs. RA model mice treated with DAH solution has showed a significant decrease of autoantigen-specific (C-II-specific) T cell proliferation in LNCs compared with control group(control group - non-Tx BALB/c, ** $p < 0.01$, Student's t-test).

Ⅳ. Discussion

According to Sinnongbonchogyeong(神農本草經), it is well documented that Nokyong(鹿茸) is sweet and warm in nature, and it has multiple pharmaceutical properties including blood purification, replenish vital essence, strengthening of muscle and bone along with anti-aging property. Afterward it has been prescribed as the most popular form of invigorating the kidney-yang, aphrodisiac in traditional Korean medicine[12].

Rheumatoid arthritis(RA) is considered to belong to the cathegory of Bi(痺) in traditional Korean medicine. According to Hoangjenaegyeong(黃帝內經), it is documented that Bi is being caused by Pung(風), Han(寒), Seub(濕), and many literatures has recorded it's symptomatology and treatment ever since. Bi has been classified according to it's etiology into following 7 kinds of Bi such as Pungbi(風痺), Hanbi(寒痺), Seubbi(濕痺), Yeolbi(熱痺), Eohyeolbi(瘀血痺), Dambi(痰痺) and Heobi(虛痺), and it is also classified according it's symptomatology into 3 kind of Bi such as Haengbi(行痺), Tongbi(痛痺) and Chakbi(着痺). It is also classified according it's location of lesion. In treatment protocols, eliminating the Pung, Han, Seub in the case of mild symptoms and eliminating the Eohyeol(瘀血) and Dameum(痰飮) in the case of chronic disorder is preferred as treatment method. If the patients are weak, it is recommended additionally to replenish them vital essence and invigorate the kidney-yang[13].

RA is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the bone tissue cells, and systemic production of autoantibodies to the collagen[14]. MHC class II molecules encounter and bind antigenic peptides as class II-peptide complexes on the cell surface of APCs for recognition by CD4+ T cells[15-17]. The molecular mechanisms leading to formation of class II-peptide complexes and presentation of antigen on the cell surface begin with synthesis of class II αß heterodimers in the endoplasmic reticulum. These class II αß heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii)[18]. Inhibition of Ii degradation in B lymphoblastoid cells and murine spleen cells induces accumulation of class II-associated Ii fragments and inhibition of class II-peptide formation[19]. Selective inhibition of the proteases responsible for both these degradative processes is a potential mechanism for modulating the immune response[20]. Several lysosomal proteases have

been implicated in the processing of Ii and antigenic peptides. Cathepsin B, the most abundant lysosomal cysteine protease, has been tied to Ii degradation using purified class II-Ii complexes[21]. Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ii form p41[22]. Cathepsin S containing potent endoproteolytic activity is highly expressed in the spleen and professional APCs and other class II-positive cells and is inducible by IFN-γ[23]. In mouse splenocytes, inhibition of cathepsin S also induces buildup of Ii breakdown products and attenuation of class II-peptide association, although the extent of this effect appears to be haplotype-dependent[24].

DAH solution is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the DAH solution modify the clinical status of RA are not well understood. Previously, our DAH solution inhibited production of IL-1β and TNF-α from macrophags in response to in vivo stimulation with bacterial lipopolysaccharides when the extract was administered into mice once a day for 7 days[6], suggesting that the DAH solution administered orally into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. Therefore, in this study, we examined the influence of DAH extract on cellular immune responses by using mouse collagen induced arthritis(CIA), an experimental model for RA. The present results clearly demonstrated that the extract strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro.

It was reported that a cleavage product of type-II collagen functions as an important autoantigen in the pathogenesis of RA in both an animal model and humans[14]. In a similar study, the observation that thyrocytes express MHC class II molecules in Graves thyroiditis suggested that nonlymphoid cells that express MHC class II molecules provoke autoimmune responses by presenting autoantigens[25]. Autoantigen-stimulated (C-II-stimulated) proliferative T cell response using bone tissue cells was clearly inhibited by the incubation with DAH solution. A large proportion of class II-expressing (I-Aq-expressing) cells was observed on bone tissue cells from RA model mice, and MHC class II molecule can be stably induced by IFN-γ stimulation on bone tissue cells (Cartilage tissue and synovial cells) from syngeneic control mice.

From the presnet results, it is possible for us to conclude that the bone tissue cells may function, at least in part, as autoantigen-presenting cells in the development of murine RA, and that inhibition of cathepsin S and L prevents autoantigen presentation and subsequent peptide binding by class II molecules. MHC class II molecules bind a diverse array of peptides derived from the endocytic pathway and present them to CD4+ T cells. APCs have a pool of active class II molecules on their surface that can quickly load peptides from the extracellular milieu for T cell presentation[8].

In this study, the treatment with DAH solution was effective in preventing the development of autoimmune lesions in the bone tissue cells of the RA model mice. Almost entire remissions of RA were induced in the bone tissue cells by the DAH solution treatment. In addition, DAH solution-treated mice showed a significant downregulation of autoantigen-specific (C-II-specific) T cell response and Th1 cytokine expressions. Although we could not exclude the possibility that the DAH solution treatment in vivo

inhibits only cathepsin S and L that other enzymes could be involved, these results indicate that DAH solution plays an important role in preventing autoantigen presentation that is followed by inhibition of autoimmunity.

Taken together out of our experiments, DAH solution might be a useful tool for the treatment of RA. However, biochemical and metabolic analysis of the constituents of DAH solution have to be analysed in further delineating its mechanisms of action in arthritis. Selective inhibition of cysteine protease cathepsin S and L by DAH solution may have important therapeutic potential in modulating class II-restricted autoimmune processes.

V. References

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