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원 저

Inhibitory Effect of Deer Antler Aqua-acupunture (DAA) on Cathepsin S Activity and Rheumatoid Arthritis in Rats

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국문초록

생쥐에서 녹용약침액이 자가항원제시형 Cathepsin S 활성의 저해와 류마티스 관절염 억제에 미치는 효과

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목적 : 시스테인 단백분해 효소인 cathepsin는 인간과 생쥐의 항원제시세포에서 Ⅱ형 주적합항원 불변사슬(MHC class Ⅱ invariant chain)의 분해에 관여한다. 본 연구는 녹용 약침액이 류마티스 관절염 생쥐 모델의 골조직(연골과 활액) 유래 cathepsin 활성에 미치는 영향을 검정하였다.

방법 : 관절염 동물모델은 BALb/c계 생쥐를 생후 3일에 흉선 적출(3d-Tx)을 하여 만들었다. 동물모델의 골조직, 임파절세포, 비장 등을 녹용처치군과 대조군으로 나누어 cathepsin의 활성도 및 자가항원 특이(C-Ⅱ-specific) T-세포의 활성도를 비교 분석하였다.

결과 : 각 장기에서 cathepsin S의 활성은 녹용약침 처치군에서 농도 의존적으로 유의성 있게 억제되었고, T-세포 특이 자가항원 반응은 녹용약침 처치군의 임파절 세포에서 유의성 있게 억제되었다. 그리고 T-세포 특이 자가항원 반응의 불활성화에는 녹용 10~20ug/ml의 용량으로 충분하였다.

결론 : 이러한 실험결과는 녹용 약침액이 cathepsin S 를 선택적으로 억제시켜 류마티스 관절염과 같은 자가면역 질환에 유효한 치료약물로 사용될 수 있음을 시사한다.

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Key words : Bone tissue cells, Cathepsin S, Invariant chain, Rheumatoid arthritis, Nokyong

I. Introduction

Unossified horn or pilose antler cut from deer which belong to the Cervidae is generally termed "Nokyong". Nokyong is one of the most famous Korean traditional medicines and has been considered to possess sexual-reinforcing and anti-aging actions. Thus, deer antler aqua-acupunture(DAA) has been used invigorate the kidney-yang(腎陽), replenish vital essence and blood and strengthen muscle and bones in a traditional Korean medicine. Water extract of DAA, prepared from the pilose antler of *Cervus korean* TEMMINCK var. mantchuricus Swinhoe(Nokyong), a traditional immunosuppressive and immuno-activating Korean aqua-acupuncture, have sometimes been compounded in recent Korean commercial restoratives, although little is yet known about the pharmacological effects or active ingredients. Extract from DAA 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 an equilibrium between the processes of tissue synthesis and degradation during cartilage and bone remodelling. It is generally accepted that proteolytic enzymes are involved in the catabolic aspect of normal tissue remodelling[3,4)], and that altered activity of these enzymes is responsible for cartilage destruction and bone erosion associated with degenerative disorders such as and rheumatoid arthritis(RA). RA has been classified as several different names in Korean oriental medicine. Those are Bi(痺), RoukJulPoong(歷節風), Baek-HoRoukJulPoong(白虎歷節風), TongPoong(痛風) and RyuPoongSpSung arthritis(類風濕性關節炎)[5)].

Rheumatoid arthritis(RA) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the bone tissue cells (Cartilage tissue and synovial cells), and systemic production of autoantibodies to the collagen[6)]. 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[7)-9)]. 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 α ß heterodimers in the endoplasmic

reticulum. These class Ⅱ αß heterodimers associate early during biosynthesis with a type Ⅱ membrane protein, the invariant chain(Ⅱ)[10]. 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[11]. Selective inhibition of the proteases responsible for both these degradative processes is a potential mechanism for modulating the immune response[12]. 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[13]. Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ⅱ form p.41[14]. 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[15]. 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[16].

We have screened specific inhibitory effects of Nokyng in vivo as well as in vitro[17,18]. 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, an 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(Cartilage tissue and synovial 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 inhibition of cathepsin S by traditional medication such as Nokyong has important functional consequences in modulating the autoimmune response.

Ⅱ. Materials & Methods

1. Materials

DAA tablets(D-13-23), a water extract of DAA were purchased from Kyungju Oriental Medical Hospital, Dongguk University, Kyungju city, Kyungpook, Korea as an i.p injection grade for human. Each Tablet contained 100㎍ of the extract. For i.p. injection into rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50㎍/10㎕.

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 were purchased from Genetic Resources center, Korea Research Institute of Bioscience and Biotechnology, (Taejon, 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(λ_{ex} 380㎚, λ_{em} 440㎚). 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(2.5m mol/l) were prepared in 10% ethanol.

Bone tissue cells(Cartilage tissue and synovial 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 3,500g 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., Taejon, Korea) as substrate at pH 5.0 for cathepsin B, with Z-Phe-Arg-methyl coumarylamide for cathepsin L, and using the method described [23] for cathepsin S. Cathepsin B or cathepsin L : 50mmol/l CH_3COOH/CH_3COONa buffer pH 5.5, 2mmol/l DTT, 0.25mmol/l CBZ-Phe-Arg- AMC(cathepsin B+L) or 0.25mmol/l CBZ-Arg-Arg-AMC(cathepsin B only) ; cathepsin H : 50mmol/l KH_2PO_4/K_2HPO4 buffer pH 6.0, 1mmol/l DTT, 0.5mmol/l puromycin, 0.25mmol/l Arg-AMC were also used.

Assay of cathepsin D activity was based on the spectrophotometric procedure of Pennington[19] : 50mmol/l $CH_3COOH/CH3COONa$ buffer pH 3.5, 1mmol/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 2,000g for 10 min, and the absorbency of acid soluble peptides determined at 280㎚. Assay blanks were run as above.

The reaction was initiated by addition of

substrate(10μM final concentration) after pre-incubation with the test compound for 3 minutes at 37℃. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured in a fluorescence spectrophotometer(Shimazu Co., Tokyo, Japan). Emission at 460nm was measured with excitation at 370nm.

4. In vivo tretment with Nokyong

We examined the in vivo therapeutic effects of Nokyong in a murine model for RA. Nokyong dissolved in PBS, was administered intraperitoneally into model mice at doses of 50 μg 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[20]. These slides were scored by three independent, pathologists 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-mercapto-ethanol. For proliferation assay, a total of 5×10^6 cells per well were cultured for 72 hours under stimulation of type-II collagenn(C-II, 10μg/mℓ), ovalbumin(OVA)(10μg/mℓ), and concanabalin A (ConA)(5μg/mℓ), and pulsed with 1 μCi/well of [^{3}H]thymidine(NEN Life Science Products Inc., Boston, Massachusetts, USA) during the final 20 hours of the culture. [^{3}H] 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 (synovial and cartilage 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-μm nylon mesh filter and were redigested for 30 minutes by the same digestion procedure, and then the digest suspension was passed through a 100-μm nylon mesh filter. Adherent cells, after culture in MEM containing 10% FBS for 24 hours at 37℃, 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 col-

lagen was analyzed by immunoblotting and ELISA as described : Blood was collected individually once a week from tail vein, and the serum anti-CII antibodies titer was measured by a solid-phase enzyme-linked immunosorbent assay(ELISA). Alternatively, blood was obtained from the rats by cardiac puncture under ether anesthesia, after which serum antibody levels to CII were measured by the ELISA as described [21]. Wells of 96-well microtiter plates(No. 3912, Becton Dickinson, Oxnard, CA) were coated with 100㎕ of CII at a concentration of 25㎍/㎖ 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㎕ 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㎕/well) in duplicate, and incubated for 2 h at room temperature. After washing, 100㎕ 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 ㎕/well. After incubation for 45min, 100㎕ of substrate solution(o-phenylenediamine dihydrochloride)(Sigma Co) was added. The reaction was stopped by adding 100㎕ of 4 N H_2SO_4. The optical density(OD) of absorbance at 490㎚ was measured with an ELISA reader of SPECTRAmax microplate spectrophotometer (Molecualr Devices, Sunnyvale, CA, USA). The quantity of IgG anti-CII antibody as anti-collagen titer was expressed as mg/100㎖ of serum by comparison with standard curves obtained from an affinity-purified rat anti-CII 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 AFN 303-318 peptide(described below). In vitro effects of Nokyong were examined by the preincubation with Nokyong(10~20㎍/㎖) 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 Nokyong on endogenous activities of cathepsin in tissue samples

We first investigated endogenous activities of cathepsin S, cathepsin L, and cathepsin B in tissue samples of the bone tissue(cartilage and synovial cells), regional lymph nodes, and spleens from RA model mice and controls.

As shown in Fig. 1, the activities of both cathepsin S and cathepsin L were significantly higher in the bone tissue(cartilage and synovial cells), lymph nodes, and spleens from model mice than those of controls. No difference in activities of cathepsin B was found in any organ from these mice. Significantly inhibitory effect of Nokyong on cathepsin S were observed in each organ in a dose-dependent manner. Moreover, we confirmed that cathepsin S activity in each organ was clearly inhibited by Nokyong <Fig. 2>.

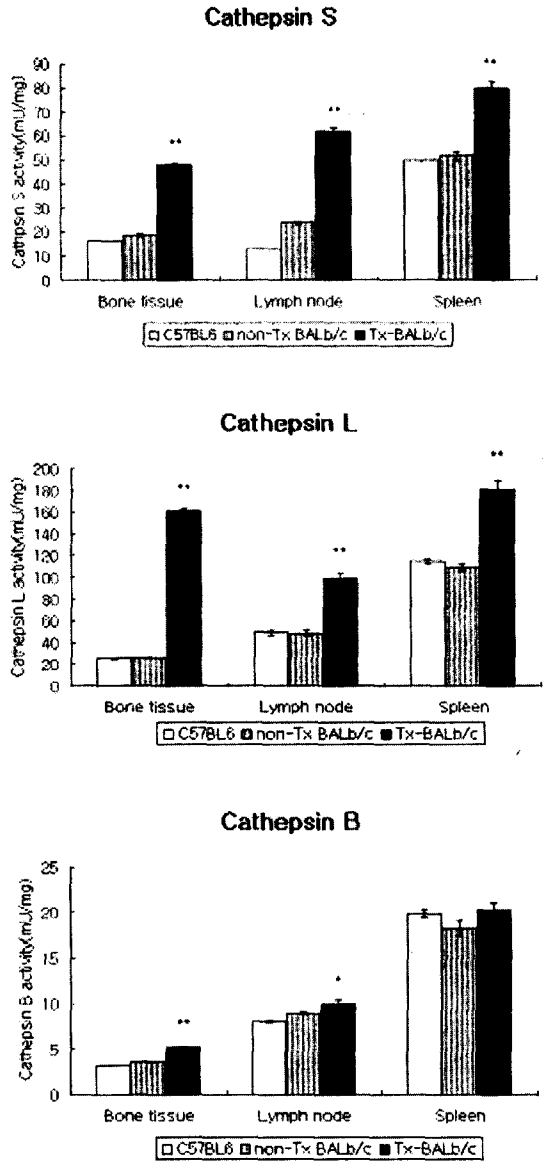

Fig. 1. Endogenous cathepsin activities in tissue samples from RA model mice and controls.

The activities of both cathepsin S and cathepsin L were significantly higher in the bone tissue(cartilage and tissue-synovial cells), regional lymph nodes, and spleens from model mice than in those from controls (* $P<0.05$, ** $P<0.01$, Student's t-test). Cathepsin activities were assayed as described in Methods.

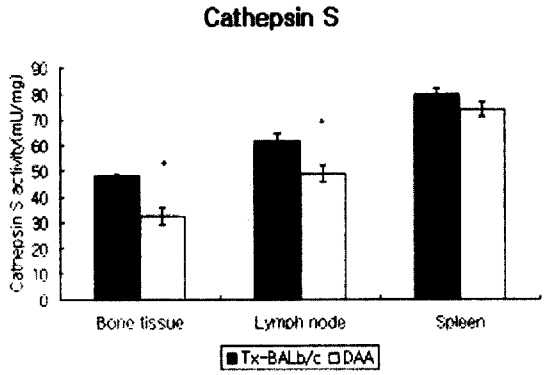

Fig. 2. Effect of Nokyong administration on endogenous cathepsin S in tissue samples from RA model mice.

The activities of cathepsin S, which were significantly higher in the bone tissue(cecartilage tissue-synovial cells), regional lymph nodes, and spleens from model mice than in those from controls(* P<0.05, Student's t-test), were significantly decreased.

2. Inhibitory effect of a Nokyong as Cathepsin on proliferative T-cell esponse to autoantigen

To address the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the LNCs and spleen cells from model mice and controls. 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>. No significant difference was observed in the proliferative response stimulated with OVA(10㎍/㎖), however Con-A(5㎍/㎖) decreased the proliferative response with unknown reason among these mice. We examined the inhibitory effects of Nokyong against autoantigen-specific T cell responses in vitro. In regional LNCs, but not in spleens, from model mice, a significantly inhibitory effect of Nokyong was observed in a dose-dependent manner<Fig. 4>. Indeed, the Nokyong at 10 to 20ug/㎖ concen-

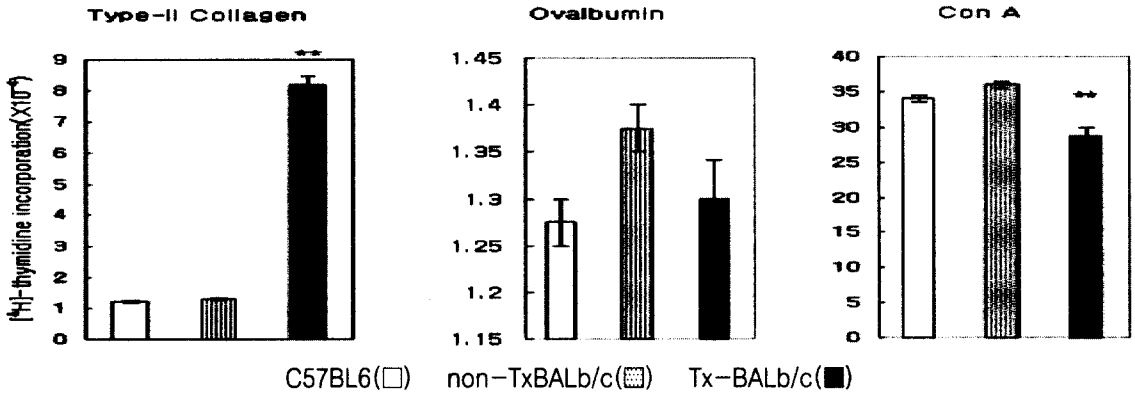

Fig. 3. Detection of proliferative T cell response of LNCs from RA model mice to organ-specific autoantigen(C-Ⅱ).

No differences was found in OVA(10㎍/ml), however Con-A(5㎍/ml) conferred significant difference(P <0.01, Student's t-test).**

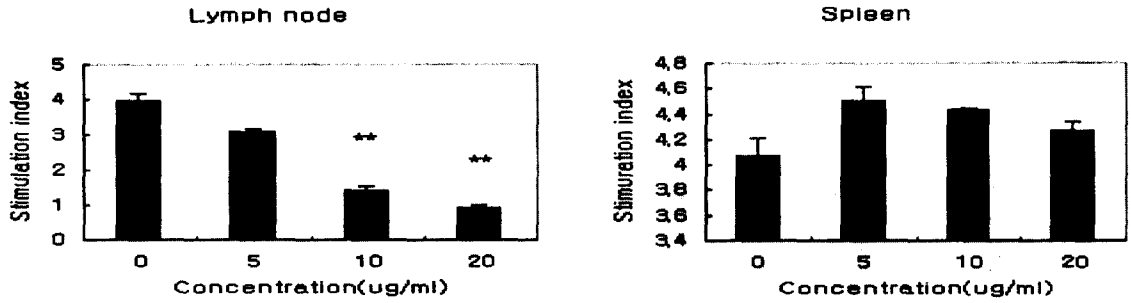

Fig. 4. In vitro preventive effect of proliferative T cell response of LNCs, but not spleen cells, to C-II by Nokyong in a dose-dependent manner.

Data are expressed as stimulation indices±SEM. Three experiments from each group were performed at 8 weeks of age, and the mean values of index were statistically significant(** P<0.01, Student's t-test).

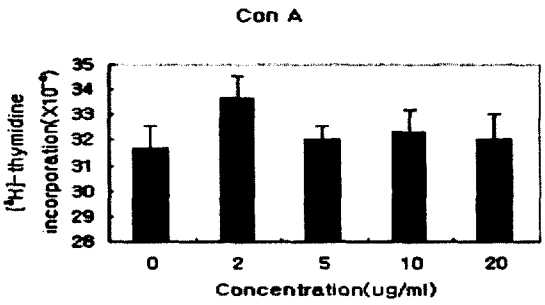

Fig. 5. Nokyong at 1 to 20ug/ml concentrations do not block T cell proliferation to Con A(5 μg/ml).

trations do not block T cell proliferation to ConA<Fig. 5>. These findings indicated that the dose of Nokyong 10 to 20ug/mℓ was sufficient to inactivate the autoantigen-specific T cell responses in vitro.

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

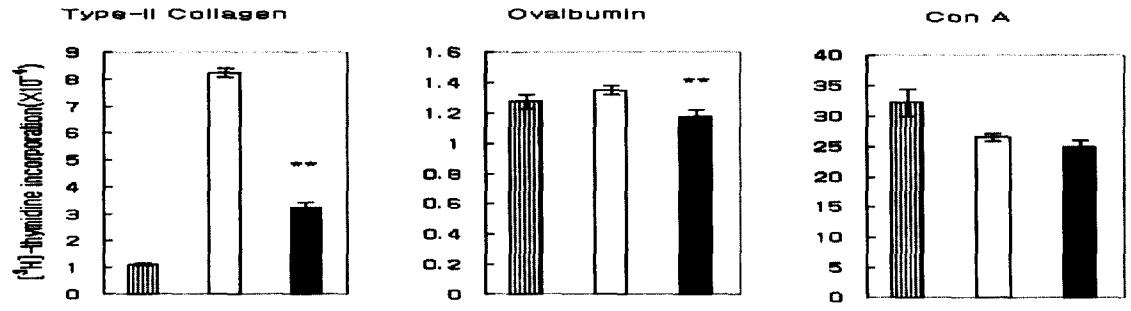

Fig. 6. RA model mice treated with Nokyong showed a significant decrease of autoantigen-specific(C-II-specific) T cell proliferation in LNCs, compared with controls.

Data are expressed as cpm per culture in triplicate(** P<0.01, Student's t-test).

We next examined the in vivo therapeutic effects of Nokyong in a murine model for RA. In addition, autoantigen-specific(C-Ⅱ-specific) T cell response was significantly inhibited in LNCs from Nokyong-treated mice <Fig. 6>. These results strongly suggest that Nokyong plays an important role in preventing autoantigen-recognizing T-cell proliferation.

Ⅳ. Discussion

DAA extract is widely used in the chronic management and the treatment of RA, particularly, in Korea. However, the mechanism by which the DAA extract modify the clinical status of RA are not well understood. Previously, our DAA extract 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[17], suggesting that the DAA extract 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 DAA extract on cellular immune responses by using rat CIA(collagen induced arthritis), 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[6]. 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[22]. Autoantigen-stimulated(C-Ⅱ-stimulated) proliferative T cell response using bone tissue cells (Cartilage tissue and synovial cells) was clearly inhibited by the incubation with Nokyong. A large proportion of class Ⅱ-e xpressing(I-Aq-expressing) cells was observed on bone tissue cells(Cartilage tissue and synovial 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 that the bone tissue cells(Cartilage tissue and synovial cells) may function, at least in part, as autoantigen-presenting cells in the development of murine RA, and that inhibition of cathepsin S 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[23].

In this study, the treatment with Nokyong was effective in preventing the development of autoimmune lesions in the bone tissue cells(Cartilage tissue and synovial cells) of the RA model mice. Almost entire remissions of RA were induced in the bone tissue cells (Cartilage tissue and synovial cells) by the Nokyong treatment. In addition, Nokyong 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 Nokyong treatment in vivo inhibits only cathepsin S and that other enzymes could be involved, these results indicate that Nokyong plays an important role in preventing autoantigen presentation which is followed by inhibition of autoimmunity.

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

Ⅴ. References

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