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원저

Effects of The pilose antler of Cervus korean TEMMINCK var. *mantchuricus Swinhoe*(DAS), herbal acupuncture solution on suppression of collagenolysis and bone resorption in mouse calvarial osteoblasts

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국문초록

녹용약침액이 mouse의 두개골 골아세포에서 collagen용해와 골재흡수에 미치는 효과

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본 실험에서는 녹용 약침액의 항 골재흡수 속성을 조사하였다. PTH, 1,25$(OH)_2D_3$와 IL-1을 각각 골재흡수 인자로 사용하여 생쥐의 두개골에서 osteoblast 세포를 격리, 배양, 그리고 자극시켰을 때 collagenolysis의 증가를 보였다. 두 가지를 동시에 사용한 결과, IL-1은 골재흡수성을 촉진시키고 재 흡

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수력을 생산하였다. In vitro에서의 세포독성 결과는 1-200 μg/ml의 녹용 약침액 농도 분포에서 무세포독성을 보였다. 또한 녹용 약침액은 생쥐의 두개골 골아세포 내에서 PTH (2 units/ml), IL-1α (1 ng/ml), 1,25$(OH)_2D_3$ (10 ng/ml), IL-1α 및 IL-1β로 인해 유발된 collagenolysis에 대해서 대항하는 보호활동성을 나타내었다. 녹용약침액은 IL-1α 와 IL-1β로 인해 유발된 collagenolysis에 대항하는 보호활동성을 지녔다. DAS는 IL-1α- 와 IL-1β로 인해 촉진된 골 재 흡수력을 억제하는 효과를 보였다. 이와 같은 결과는 녹용약침액이 골다공증과 연관된 질환에 대해서 매우 안정적인 임상적 사용이 가능한 것을 관찰할 수 있으므로 추후 이와 관련한 지속적인 연구가 필요할 것으로 사료되었다.

Key words : pilose antler of Cervus korean TEMMINCK var. *mantchuricus Swinhoe*(DAS); bone resorption; osteophoresis; calvarial bone cells

I. Introduction

It is well known that Korean herbal medicine, **pilose antler of *Cervus korean* TEMMINCK var. *mantchuricus Swinhoe (DAS)***, is effective for the treatment of bone resorption according to the ancient Korean medicinal literature [1]. The DAS is medicines applied in Korea as an effective biological response modifier for augmenting host homeostasis of body circulation [1]. This DAS medicine has been shown to express diverse activities such as immunomodulating, and anti-inflammatory effects [2-3]. Thus, it still occupies an important place in traditional Oriental medicine.

Bone resorption is known to be affected by both circulating and locally produced factors. Parathyroid hormone, vitamin D metabolites, and calcitonin are the major circulating hormones affecting bone resorption [4-5]. The cytokines of inteleukin-1, tumor necrosis factor, epidermal growth factor, transforming growth factor, and certain prostaglandins are locally produced factors which have been shown to stimulate bone resorption in vitro. These locally exsited factors are all expected to have local effects on bone. The bone resoprtion is sometimes mediated by the synergistic activities by those factors [6].

To examine the inhibitory effect of DAS extracts on the bone resorption and collagenolysis induced by PTH, 1,25$(OH)_2D_3$, IL-1α and IL-1β in the mouse calvarial bone cells, we have assayed the inhibitory activities of DAS extracts. The assays for the inhibition of bone resorption and collagenolysis are composed of *in vitro* cytotoxicities on mouse calvarial bone cells, collagenolysis, gelatinase activities, and bone resorption activity with a pretreatment and posttreatment of the DAS. From the the results, it was concluded that the DAS are highly stable and applicable to clinical uses in osteoresorption. It is

generally known that inflammation induces bone resortion. Therefore, anti-bone resorption activity may be assessed by the effect on osteoblastic cells. The present paper reports the effect of DAS on cytokine-induced experimental bone resortion in mouse calvarial cells. The assays for the inhibition of bone resorption and collagenolysis are composed of *in vitro* cytotoxicities on mouse calvarial bone cells, collagenolysis, and bone resorption activity with a treatment of the DAS.. From the the results, it was concluded that the DAS is highly applicable to clinical uses in bone resorption.

II. Materials and Methods

1. Materials

The extracts of DAS, which was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University College of Oriental Medicien (Kyungju, Japan). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Other radiochemicals were obtained from New England Nuclear Corp. (Boston, MA). Tissue culture media and reagents, Fetal bovine serum (FBS) were from Gibco (Chagrin Falls, OH). Recombinant pure human IL-1β (specific activity 5 x 10^5 U/mg) was our deposit [10] was obtained from Genzyme Corp. (Cambridge, MA, USA).

2. MTT cytotoxicity of the DAS on the isolated mouse calvarial bone cells

Cytotoxicity of DAS on the isolated calvarial cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-formazan assay, as foloows: the cell suspension was plated (200 μl; 1~2 x 10^4 cells/ well) in a 96 well-microculture plate (flat bottom; Falcon 3027) (Becton Dickinson and Company, New Jersey, USA). After 24 h culture, 30 μl of varying concentrations of each sample solution was added to the wells and cultured for 3 days. Finally, 50 μl of MTT solution (5 mg/ml in DMEM or RPMI 1640 medium) was added to the wells and further incubated for 4-6 h. After incunation, the culture supernatants were discarded by aspirating and then 250 μl of dimethyl sulfoxide (DMSO) was added. The optical density (O.D) was measured in 50 mM glycine buffer with enzyme-linked immunosorbent assay (ELISA) using by a microplate reader MPR-A4 at 540 nm. The mean value of O.D of 5-6 wells was used for the calculation of the % cytotoxicity and the equation was as follows: % cytotoxicity = (1-O.D treated well/O.D control well) x 100.

3. Osteoblasts isolation and culture

Mouse calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, as described for rat osteoblasts [11-12]. Explants of mouse calvarial bone were cultured and the cells obtained have been routinely characterized and shown to express an osteoblast-like phenotype in culture. The population released during the last three digestions was highly enriched in cells

expressing two markers of the osteoblast lineage, alkaline phosphatase and osteocalcin [13]. Cells released by collagenase digestions were washed and grown to confluent in 75㎠ culture flasks (Falcon) in (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco, BRL, Bethesda, MD, USA). Incubations were carried out at 37℃ in a humidified atmosphere of 5% CO_2/95% air; the medium was changed every 2-3 days. Cells were grown to confluence at 37℃ and cultured in duplicate or triplicate wells for an additional 24 h in serum-free medium supplemented with Polymixin B sulfate to prevent endotoxin effects prior to treatment.

4. Measurement of collagenolysis with PTH, 1,25(OH)$_2$D$_3$, IL-1α and IL-1β

Calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, as for mouse osteoblasts [7]. Cells released by collagenase digestions were washed and grown to confluent in 75㎠ culture flasks (Falcon) in DMEM supplemented with antibiotics and 10% FCS. Incubations were carried out at 37℃ in a humidified atmosphere of 5% CO_2/95% air; the medium was changed every 2-3 days. Radiolabelled collagen films were prepared as described [8]. Aliquots of [^{14}C]acetylated collagen [mouse skin type I; 150μg in 300 μl of 10 mM phosphate buffer (pH 7.4), containing 300 mM NaCl and 0.02% sodium azide] were dispensed into tissue culture wells and dried at 37℃. The collagen was washed twice with sterile distilled water and once with DMEM prior to use. First passage cells (10^5/well) were settled onto the collagen films and cultured in 1 ml DMEM with 10% FCS for 24h. After a wash in serum-free DMEM, the cells were cultured for either 72 or 120 h in DMEM (500μl) with 2% acid treated mouse serum (this contains no α$_2$-macroglobulin or other detectable proteinase inhibitors). Cells were stimulated with either PTH (2 units/ml), or MCM (5%, v/v), or IL-1β, or IL-1α (1 ng/ml) or 1,25(OH)$_2$D$_3$(10 ng/ml). PTH (1-84) and rhIL-1α were supplied by Funabashi Co., (Tokyo, Japan). MCM was partially purified from cultured pig leucocytes on Ultrogel ACA-54 as described [9]. At the end of the culture period the media were centrifuged (10 min, 1200 xg) to remove any collagen fibrils, and the radioactivity released during collagen degradation quantified by liquid scintillation counting.

5. Effect of DAS on PTH, 1,25(OH)$_2$D$_3$, IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells

The assay was carried out to assess the anti-collagenolysis activity of DAS (100 μg/ml) on PTH, 1,25(OH)$_2$D$_3$, IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells [14], as follows: The mouse calvarial osteoblast cells were treated with PTH, or 1,25(OH)$_2$D$_3$, IL-1α and IL-1β to induce the collagenolysis for 56 hr, and the treated cells were further treated with DAS for 16 hrs, and the reduced collagenolysis was assayed.

6. Analytical methods

Protein content was determined by the method of Lowry [15] with bovine serum albumin as the standard. Protein in the cell culture medium was routinely followed by the absorbance at 280 nm [16].

7. Bone resorption assay

The fetal mouse long bone organ tissue culture system was based on that described by Raisz [17]. Fetal bones were labeled with ^{45}Ca by injecting the mother with 200 μCi ^{45}Ca (NEN, Boston, MA) on the eighteenth day of gestation. Radii and ulnae bone shafts were obtained from 19 day fetuses by microdissection. The shafts were cut just beyond the calcified zone and therefore contained short lengths of cartilage at the ends. Bones were cultured on sunk Millipore filter dots in 24-well Limbro plates. The shafts were first cultured in 0.5 ml BGJ_b medium(Gibco Laboratories, Grans Islan, NY) containing 1.0 ml/ml bovine serum albumin, 100 units/ml penicillin G, and 1 μg/ml polymyxin B for 1 day to reduce exchangeable ^{45}Ca. One bone from a fair (right and left radii or right and left ulnae from a single fetus) was then transferred into medium containing agonist(s) (treatment) and the contralateral bone was placed into identical medium without agonist(s) (control). A typical test group consisted of 5 pairs of bones. Bones were cultured for 5 days in a 95% air / 5% CO_2 incubator at 37℃ and 95% humidity with one change of media after 2 days. The percentage of ^{45}Ca released from a bone into the medium during the 5-day culture was determiend by measuring the radioactivity in mediuml, medium 2, and the trichloroacetic acid solubilized bone using a liquid scintillation counter. Stimulated resorption was expressed as the paired difference bwtween treatment and control bone percent ^{45}Ca released from during the 5-day culture. Dead bone ^{45}Ca release in this system was apprximately 10%. BGJ_b control ^{45}Ca release was 16-20% and maximum IL-1b ^{45}Ca release was 60-80%. Since "stimulated" release is expressed as the mean difference between paired BGJ_b control bones (C%) and treated bones (T%), the T%-C% for an inactive treatment is zero, and a maximum IL-1β response is approximately 40-60%. Each bone was labeled with approximately 20,000 CPM ^{45}Ca.

8. Statistics

Statistical differences between treatments were determined using analysis of variance.

Ⅲ. Results & Discussion

1. MTT assay of DAS on mouse calvarial bone cells

The results of in vitro cytotoxicities showed that DAS has no any cytotoxicities in concentrations of 1-200 μg/ml and furthermore there is no any cytotoxicity even in concentration of 30 μg/ml on mouse calvarial bone cells (Table 1). However, in higher concentration of the DAS, the MTT reduction observed and the degree of inhibition was increased in a dose-dependent manner from 1.0 mg/ml concentrations. In contrast, 200 μg/ml of LPS, a cytotoxic and inflammatory control reagent, showed the severe cytotoxicity on the mouse calvarial bone cells, resulting in 75% of cell death of the cells. These results indicated that the DAS are highly stable and applicable to clinical uses. However, for 100 μg/ml of each extract was used for the next experiments.

Table 1. MTT assay of DAS on mouse calvarial osteoblasts

	DAS (μg/ml)					
	0	5.0	10.0	100	200	LPS
OD at 560nm	0.74±0.11	0.57±0.06	0.73±0.05	0.71±0.06	0.68±0.06	0.12±0.01

As a negative control, 200 μg/ml LPS gave significant inhibition of activity. Each point represents the mean± s.d. of 5 experiments from separate joints.

2. Effects of DAS on PTH, $1,25(OH)_2D_3$, IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells

When calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, and cells were stimulated with either PTH (2 units/ml), or MCM (5%, v/v), or IL-1α (1 ng/ml) or $1,25(OH)_2D_3$ (10 ng/ml), IL-1α and IL-1β. Then, the radoactivity released during collagen degradation was quantified by liquid scintillation counting and collagen degradation was expressed as a percentage radioactivity released from the films (mean ± S.E.) for five wells. There were small but statistically significant increase in collagenolysis with PTH and $1,25(OH)_2D_3$ treatment after 56 hrs. (Table 2, 3) Also, DAS was tested for whether it could protect against PTH (2 units/ml),or IL-1α (1 ng/ml) or $1,25(OH)_2D_3$ (10 ng/ml), IL-1α and IL-1β -induced collagenolysis in the mouse calvarial cells (Table 2-4). Cell viability was not significantly affected by treatment with the indicated concentration of DAS alone, as examined also in MTT assays.

Table 2. Lysis of ^{14}C-labelled type I collagen films by mouse calvarial osteoblasts and effects of DAS on PTH-induced collagenolysis

			DAS			
	Control	PTH	1h	4h	8h	16h
% Lysis $[^{14}C]$Collagen	41.6±3.2	65.3±4.6	66.3±6.2	65.7±4.7	61.7±4.6*	55.4±3.3**

Collagen degradation was expressed as a percentage radioactivity released from the films (mean ± S.E.) for four wells. The cells, which were treated with PTH for 56 h, were further treated with DAS and then PTH-induced collagenolysis was assayed after 16 hrs.

*, Significantly greater than control cultures, $P<0.05$

**, Significantly greater than PTH cultures, $P<0.001$. PTH, parathyroid hormone treatment

Table 3. Lysis of ^{14}C-labelled type I collagen films by mouse calvarial osteoblasts and effects of DAS on $1,25(OH)_2D_3$-induced collagenolysis

			DAS			
	Control	$1,25(OH)_2D_3$	1h	4h	8h	16h
% Lysis [^{14}C]Collagen	36.7±2.4	80.8±9.2	82.8±6.3	76.8±5.2	76.1±6.4*	67.3±7.2**

Collagen degradation was expressed as a percentage radioactivity released from the films (mean ± S.E.) for four wells. The cells, which were treated with 1,25(OH)2D3 for 56 h, were further treated with DAS and then 1,25(OH)2D3-induced collagenolysis was assayed after 16 hrs.

*, Significantly greater than 1,25(OH)2D3 cultures, $P<0.05$

**, Significantly greater than control cultures, $P<0.001$

3. Effects of DAS on IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells

We tested the ability of recombinant human IL-1 (IL-1α; 0.1-20.0 ng/ml) to stimulate collagen degradation by the cells; maximal collagenolysis (69%) was again only achieved after 56 hrs with the optima dose of 10 ng/ml (Table 4). Also, IL-1β was tested for stimulation of collagen degradation (0.1 - 2.0 ng/ml). The maximal collagenolysis was obtained after 56 hrs with the optima dose of 5 ng/ml(Table 5). To examine the anti-collagenolysis of the DAS on IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells, various concentrations of the DAS were tested for whether they could protect against IL-1α (2 ng/ml) or IL-1β (1 ng/ml)-induced collagenolysis in the mouse calvarial cells (Table 4 and 5). Cell viability was not significantly affected by treatment with the indicated concentration of the extracts alone, as examined also in MTT assays.

Furthermore, the DAS were shown to have the protective effects against collagenolysis induced by the bone resortion agents of IL-1α and IL-1β. However, their effects were not stringent to protect the collagenolysis. Treatment of the DAS for 1 h, which by itself had little effect on cell survival, did not enhance the collagenolysis, nor significantly reduced the collagenolysis by treatment (not shown).

Table 4. Effect of DAS on IL-1α(2.0 ng/ml)-induced collagenolysis by mouse calvarial osteoblasts

	Control	IL-1α	DAS
% Lysis [^{14}C]Collagen	16.5±1.5	27.2±2.4	21.9±1.2*

Collagen degradation was expressed as a percentage radioactivity released from the films (mean ± S.E.) for four wells. The cells, which were treated with IL-1α(2.0 ng/ml) for 56 h, were further treated with DAS and then IL-1α-induced collagenolysis was assayed after 16 hrs.

*, Significantly greater than IL-1α cultures, P<0.05

Table 5. Effect of IL-1β (1 ng/ml) on lysis of ^{14}C-labelled type I collagen films by mouse calvarial osteoblasts and effects of DAS on IL-1β (1.0 ng/ml)-induced collagenolysis

	Control	IL-1β	DAS
% Lysis [^{14}C]Collagen	15.8±2.1	26.9±1.7	18.2±1.6*

Collagen degradation was expressed as a percentage radioactivity released from the films (mean ± S.E.) for four wells. The cells, which were treated with IL-1β (1.0 ng/ml) for 56 h, were further treated with DAS and then IL-1β-induced collagenolysis was assayed after 16 hrs.

*, Significantly greater than IL-1β cultures, P<0.05

4. Bone resorption activity of IL-1 and effects of DAS on IL-1α-and IL-1β-induced bone resorption mouse calvarial osteoblasts

Our result showed IL-1α is significantly less potent than human IL-1β in stimulating bone resorption. Thus, the differences in relative activity of IL-1α- and IL-1β in different assays would be not unusual. DAS was shown to have the inhibiting effects against IL-1α- and IL-1β -stimulated bone resorption and the effect of the treatment with a various concentrations of the medicinal extracts were significant (Table 6, 7).

Table 6. Responses for IL-1α-stimulated bone resorption and effects of DAS on IL-1α- induced bone resorption

	Control	IL-1α	DAS
Calcium release (T% - C%)	0.2±0.01	12.1±3.2	1.9±0.2*

Bone resorption was measured as percent release of ^{45}Ca during 5 days of culture. Each point is the mean paired difference ± S.E. for 5 treatment-control bone pairs. The cells, which were treated with IL-1α (50 ng/ml) for 6 h, were further treated with DAS and then IL-1α-stimulated bone resorption was assayed after 16 hr.

*, Significantly greater than IL-1α cultures, P<0.05

Table 7. Responses for IL-1β-stimulated bone resorption and effects of DAS on IL-1β- induced bone resorption

	Control	IL-1β	DAS
Calcium release (T% - C%)	2.1±0.2	18.2±1.5	3.6±0.42*

Bone resorption was measured as percent release of ^{45}Ca during 5 days of culture. Each point is the mean paired difference ± S.E. for 5 treatment-control bone pairs. The cells, which were treated with IL-1β (5.0 ng/ml) for 6 h, were further treated with DAS and then IL-1β-stimulated bone resorption was assayed after 16 hrs.

*, Significantly greater than IL-1β cultures, $P<0.05$

IV. Discussion

When we examined the effects of DAS on MTT reduction in mouse calvarial cells. Whereas the LDH release assay is an index of membrane damage, the MTT reduction assay reflects intracellular redox state. Thus, inhibition of MTT is not necessarily accompanied by complete cell lysis. The results of in vitrro cytotoxicities showed that DAS has no any cytotoxicities in concentrations of 1-200 μg/ml and furthermore there is no any cytotoxicity even in concentration of 30μg/ml on mouse calvarial bone cells. However, in higher concentration of the DAS, the MTT reduction observed and the degree of inhibition was increased in a dose-dependent manner from 1.0 mg/ml concentrations. These results indicated that the DAS are highly stable and applicable to clinical uses. However, for 100 μg/ml of each extract was used for the next experiments

When calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, and cells were stimulated with either PTH (2 units/ml), or IL-1α (1 ng/ml) or $1,25(OH)_2D_3$ (10 ng/ml), IL-1α and IL-1β. DAS was shown to have the protective effects against collagenolysis induced by the bone resortion agents. However, their effects were not stringent to protect the collagenolysis. The collagenolysis-induction agents has been known to increase the susceptibility of the calvarial cells against collagenolysis [18], although there are some controversies. Thus, we examined the effect of the pretreatment with a various concentrations of the DAS then treated the collagenolysis-induction agents.

We tested the ability of recombinant human IL-1α to stimulate collagen degradation by the cells. To examine the anti-collagenolysis of the DAS on IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells, various concentrations of the DAS were tested for whether they could protect against IL-1α or IL-1β-induced collagenolysis in the mouse calvarial cells. The DAS were shown to have the protective effects against collagenolysis induced by the bone resortion agents of IL-1α and IL-1β. However, their effects were not stringent to protect the collagenolysis. The collagenolysis-induction agents has been known to increase the susceptibility of the calvarial cells against collagenolysis [18],

although there are some controversies. Thus, we examined the effect of the treatment with a various concentrations of the DAS then treated the collagenolysis-induction agents. Treatment of the DAS for 1 h, which by itself had little effect on cell survival, did not enhance the collagenolysis, nor significantly reduced the collagenolysis by treatment (not shown).

It is well known in cellular and molecular aspects that IL-1α- and IL-1β have similar dose dependent responses in most biological systems [19]. However, the dose response for stimulating bone resorption differed significantly in the fetal mouse long bone organ tissue culture. Human IL-1β is aaproximately 10 times more potent than human IL-1α in stimulating bone resorption as measured by means of calcium release when each is normalized to nano gram of amounts. Analysis of covariance indicated no significant difference in the slopes of the increasing portions of the two curves. Variance ratio tests showed highly significant difference ($P<0.001$) between the adjusted (for nano gram) means for the different IL-1s. These results are similar to that obtained from fetal rat long bone organ cultures [20]. It was known that IL-1α- and IL-1β generally have the same potency and biological activity and bind to the same receptor [21]. Our result showed IL-1α is significantly less potent than human IL-1β in stimulating bone resorption. Thus, the differences in relative activity of IL-1α- and IL-1β in different assays would be not unusual. DAS was shown to have the inhibiting effects against IL-1α- and IL-1β -stimulated bone resorption and the effect of the treatment with a various concentrations of DAS were significant.

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