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A Study on Bone Formation & Osteoporosis by Taeyoungjon-Jahage Extracts

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ABSTRACT

생쥐의 calvarial osteoblast세포를 분리배양하여 gelatinase생성여부를 골흡수과정에서의 역할을 규명하기 위하여 SDS-PAGE-zymography분석을 한 결과 progelatinase-A를 항속적으로 합성하고 있음을 확인하였다. 생쥐의 osteoblasts를 골재흡수 약물인 PTH, 1,25$(OH)_2D_3$, 단핵구배양액 (MCM) 그리고 IL-1으로 자극시키면 gelatinase생산을 촉진하여 콜라겐분해가 증가되었으나, indomethacin과 dexamethasone은 생쥐의 osteoblastic세포의 collagenolysis를 저해하였다. 한편, 골재흡수에 IL-1을 생쥐태아 유래의 장골조직배양 (fetal mouse long bone organ culture)에 처리하자 IL-1 은 골재흡수를 촉진하였다. 더우기, IL-1α의 농도의존성에 대한 indomethacin과 dexametasone의 영향을 검토한 결과 직선형의 비례커브로 영향을 미쳤다. 이러한 골대사의 지견을 바탕으로 대영전-자하거의 열수추출물의 시험관내 독성검사에서 1-200 μg/ml의 농도에서는 독성이 없었으며, 또한, 300 μg/ml 농도에서도 생쥐의 calvarial골에는 독성이 없었다. 대영전-자하거 extract는 PTH (2 units/ml), MCM (5%, v/v), rhIL-1α (1 ng/ml) 1,25$(OH)_2D_3$ (10 ng/ml)처리에 대해서 그리고 IL-1α와 IL-1β-유발 collagenolysis에 대해서도 보호효과가 있었다. 대영전-자하거extract을 1시간동안 전처리와 후처리에서 콜라겐분해에 약간의 보호활성이 있었으며 IL-1α와 IL-1β에 의해 유발되는 콜라겐분해에 보호활성이 보였다. 1시간동안 전처리는 콜라겐분해를 감소시키며, 대영전-자하거 extract는 gelatinase 효소를 저해하였으며 PTH, 1,25$(OH)_2D_3$, IL-1β 및 IL-1α로 유발된 효소활성화가 저해되었다. 즉, 대영전-자하거 extracts는 IL-1α-와 IL-1β에 의해 촉진되는 골재흡수에 효과적이었으며, 비스테로이드성 항염증제제 (indomethacin 과 dexamethasone)에 의한 골재흡수방지 효과와 유사하였다. 이러한 결과는 대영전-자하거extract가 골다공증치료에 효과적임을 나타내는 것이다.

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Mouse calvarial osteoblast cells were isolated and cultured. To examine whether the cells produce active gelatinases in culture medium or not,the cells were analyzed using by zymograsphic analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We show that mouse calvarial osteoblasts in culture constitutively synthesize progelatinase-A. Then, mouse osteoblasts, which were stimulated by PTH, $1,25(OH)_2D_3$, mononuclear cell conditioned medium (MCM) and IL-1 as bone resorption agents, showed increased collagenolysis by producing the active gelatinase. However, treatment of indomethacin and dexamethasone significantly decreased those effects of collagenolysis in mouse osteoblastic cells. On the other hand, IL-1 in stimulating bone resorption was examined using fetal mouse long bone organ culture. IL-1 stimulated bone resorption and produced marked resorption when present simultaneously. Furthermore, when it was examined the effects of indomethacin and dexamethasone on the dose dependent responses of IL-1α, indomethacin and dexametasone produced a rightward shift in the IL-1 dose response curve. The results of in vitro cytotoxicities showed that Taeyoungjon-Jahage water extracts(T.Y.J-J.H.G extracts) have no any cytotoxicities in concentrations of 1-200 μg/ml and furthermore there is no any cytotoxicity even in concentration of 300 μg/ml on mouse calvarial bone cells. T.Y.J.-J.H.G. extracts had protective activity against PTH (2 units/ml), or MCM (5%, v/v), or rhIL-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. Pretreatment of the T.Y.J.-J.H.G.extracts for 1 h, which by itself had little effect on cell survival, did not enhance the collagenolysis, nor significantly reduced the collagenolysis by pretreatment. Furthermore, the medicinal extracts were shown to have the protective effects against collagenolysis induced by IL-1α and IL-1β. Pretreatment of the extracts for 1 h significantly reduced the collagenolysis. Interestingly, the T.Y.J.-J.H.G. extracts were shown to have the inhibiting effects against gelatinase enzyme and processing activity induced by the bone resortion agents of PTH, $1,25(OH)_2D_3$, IL-1β and IL-1α, with strong protective effect in pretreatment with the extracts. T.Y.J.-J.H.G. extracts were shown to have the inhibiting effects against IL-1α- and IL-1β-stimulated bone resorption and the effect of the pretreatment with a various concentrations of the medicinal extracts were significant. The inhibition extent and phenomena of IL-1-stimulated bone resorption by nonsteroidal anti-inflammatory agents of indomethacin and dexamethasone were similar to those obtained by T.Y.J.-J.H.G. extracts treatment in the mouse calvarial tissue culture system. These results indicated that the T.Y.J.-J.H.G.-water extracts are highly stable and applicable to clinical uses in osteoporosis.

I . Introduction

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[1,2]. The cytokines of interleukin-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 excited factors are all expected to have local effects on bone. The bone resoprtion is sometimes mediated by the synergistic activities by those factors[3].

Research during the past decade indicates that two interrelated mechanisms are involved in the proteolytic stages of bone resoprtion. One is osteoclast-dependent, calcitonin-sensitive and involves cysteine proteinases as well as matrix metalloproteinase (MMP) production. The other is mediated by osteoblast, is calcitonin -insensitive and involves the production of collagenase and other MMPs such as the gelatinases (type IV collagenases) and strom -elysin-1, in response to bone resorptive age -nts[4]. The latter findings support the view that osteoblasts play a major role in bone resorption by degrading the surface osteoid layer, thereby exposing the underlying mineralized matrix for osteoclastic action.

MMPs are synthesized as latent proenzyme forms that require activation and their extracellular actions are further regulated by a family od specific inhibitors, the TIMPs. The inhibitors, TIMP-1 and TIMP-2, have been extensively characterized[4]. It has also been known that human osteoblasts produce progelatinase-A (72 kDa) and TIMP constitutively. Although gelatinase-B (95 kDa) and other M MPs could be upregulated, with the assay methods used there was no evidence for the production of TIMP-2. Because animal models and cells are used extensively for bone studies, and because of the unique properties of TIM P-2, it was thought it imperative to define the TIMPs produced by non-human bone cells. The synthesis of type I collagen, a heteropol ymer composed of two α1 and one α2 chain s, can be an important marker of osteoblastic differentiation[5].

In this paper, we show that mouse calvarial osteoblasts constitutively synthesize progelatinase-A, thus, cells are still capable of degrading a type I collagen substratum under appropriate conditions. To determine whether mouse osteoblasts have the ability to degrade a type I collagen substratum mouse osteoblasts were seeded onto collagen films and stimulated. First passage cells were used in all experime nts and characterized as osteoblasts by the following criteria; staining for alkaline phosphatase positivity; osteocalcin synthesis in response to $1,25(OH)_2D_3$; type I collagen prooduction; and accumulation of cAMP in response to PTH treatment[6]. Bone resorption activity was examined using fetal mouse long bone organ culture by IL-1, together with the effects of indomethacin and dexame thasone. On the other hand, to examine the inhibitory effect of some oriental medicinal extracts on the bone resorption and collage-

nolysis induced by PTH, MCM, $1,25(OH)_2D_3$, IL-1α and IL-1β in the mouse calvarial bone cells, we have screened and assayed the inhibitory activities of T.Y.J.-J.H.G. 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 T.Y.J.-J.H.G. extracts. From the results, it was concluded that the T.Y.J.-J.H.G.-water extracts are highly stable and applicable to clinical uses in osteoporosis.

Ⅱ. Materials and Methods

1. Materials

Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, cycloheximide (CHM), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), indomethacin, dexamethasone and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). PGE_2 antibody was purchased from Immunoassay Co. (Tokyo, Japan). [^{3}H]-prostaglandin was purchased from New England Nuclear (Seoul, Korea). 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 a generous gift of Dr. S. H. Park, Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea) or was obtained from Genzyme Corp. (Cambridge, MA, USA).

2. MTT cytotoxicity of the medicinal herb extracts (T.Y.J.-J.H.G.water extracts) on the isolated mouse calvarial bone cells.

Cytotoxicity of the medicinal herb extracts on the isolated calvarial cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-formazan assay, as follows: 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 mMglycine 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[7,8]. 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[19]. 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 sup plemented with Polymixin B sulfate to preve nt endotoxin effects prior to treatment.

4. Measurement of collagenolysis with PT H, 1,25$(OH)_2D_3$, mononuclear cell conditioned medium (MCM), IL-1α and IL-1β.

Calvarial osteoblasts were isolated from neonatal BALBc mice by enzymatic digestion, as for mouse osteoblasts[4]. 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[4]. 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 rhIL-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 [4]. 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. Measurement of T.Y.J.-J.H.G.-treatment on PTH, 1,25$(OH)_2D_3$, mononuclear cell conditioned medium(MCM), IL-1α and IL-1β -induced collagenolysis in calvarial osteoblast cells.

Two different assays were carried out to assess the anti-collagenolysis activity of T. Y.J.-J.H.G.extracts (each 100 μg/ml) on PTH, 1,25$(OH)_2D_3$, mononuclear cell conditioned medium(MCM), IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells, as follows: 1) Experiment-1: The mouse calvarial osteoblast cells were treated with PTH, or 1,

$25(OH)_2D_3$, or mononuclear cell conditioned medium(MCM), IL-1α and IL-1β to induce the collagenolysis for 56 hr, and the treated cells were further treated with T.Y.J.-J.H.G. with time courses of 1, 2, 4, 8, and 16 hrs, and the reduced collagenolysis was assayed.

2) Experiment-2: The mouse calvarial osteoblast cells were initially treated with T.Y.J.-J.H.G. for 1 h and further treated with each agents such as PTH, or $1,25(OH)_2D_3$, or MCM) IL-1α and IL-1β to induce the collagenolysis for 46 and 56 hr. Finally, the reduced collagenolysis was assayed.

6. Zymography of gelatin-degrading activities of mouse osteoblasts

Samples of culture media from unstimulated and stimulated osteoblasts grown on type I collagen were electrophoresed under non-reducing conditions on a SDS-8% polyacrylamide gel containing 0.5% gelatin[4]. After electrophoresis the SDS was removed by washing (2.5% Triton X-100, 2 times for each 15 min at room temperature) and degradation was visualized by overnight incubation at room temperature in TCB buffer (100 mM Tris-HCl, pH 7.9; 30 mM $CaCl_2$; 0.02% sodium azide). Gels were stained with Coomassie blue (30 min), destained and photographed.

7. Analytical methods

Protein content was determined by the method of Lowry[7] with bovine serum albumin as the standard. Protein in the cell culture medium was routinely followed by the absorbance at 280 nm. The amount of total sugars was determined by the phenol-sulfuric acid method[8] with the glucose as the standard. The following molecular mass standards (low range, Bio-Rad Lab.) were used: phosphorylase b (95 kDa), bovine serum albumin (68 kDa), egg albumin (46 kDa), egg lysozyme (15 kDa). Acrylamide gel electrophoresis was performed in 12% (w/v) gels with or without by the method of Laemmli [9].

8. Bone resorption assay

The fetal mouse long bone organ tissue culture system was based on that described by Raisz[10]. 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 between treatment and control bone percent ^{45}Ca released from during the 5-day culture. Dead bone ^{45}Ca release in this system was approximately 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.

9. Statistics

Statistical differences between treatments were determined using analysis of variance ($P<0.05$ - 0.01).

Ⅲ. Results and Discussion

1. MTT test of T.Y.J.-J.H.G. water extracts on mouse calvarial bone cells.

The results of in vitro cytotoxicities showed that T.Y.J.,J.H.G.-water extracts have no any cytotoxicities in concentrations of 1-200 μg/ml and furthermore there is no any cyto-toxicity even in concentration of 300 μg/ml on mouse calvarial bone cells (Fig. 1~3.). However, in higher concentration of the ext-racts, 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 T. Y.J.,J.H.G.-water extracts are highly stable and applicable to clinical uses. However, for 100 μg/ml of each extract was used for the next experiments.

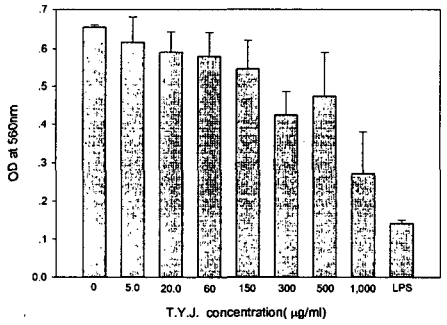


Fig. 1.

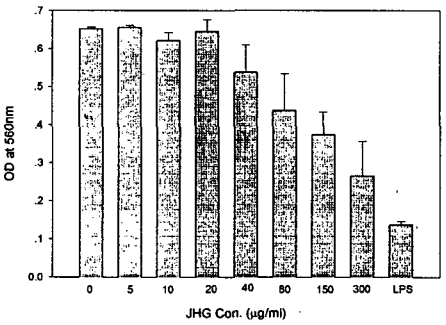


Fig. 2.

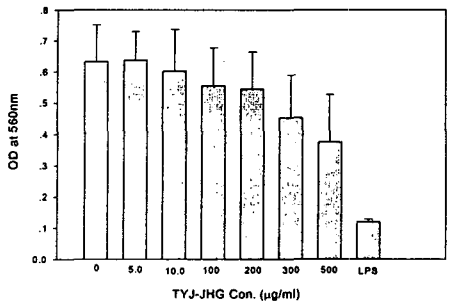


Fig. 3.

Fig. 1~3. MTT assay of T.Y.J, J.H.G and T.Y.J.-J.H.G.water extracts on mouse Calvarial osteoblasts
As a negative control, 200 μg/ml LPS gave significant inhibition of activity.

2. Effects of oriental medicinal extracts such as T.Y.J. and J.H.G. on PTH, 1,25(OH)$_2$D$_3$, mononuclear cell conditioned medium(MCM), 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 rhIL-1α (1 ng/ml) or 1,25(OH)$_2$D$_3$ (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 parathyroid hormone (PTH), 1,25-hydroxyvitamin D3 (1,25(OH)$_2$D$_3$) and MCM treatment after 56 h (Fig. 4-6).

All these medicinal extracts were 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, although there are some controversies. Thus, we examined the effect of the pretreatment with a various concentrations of the medicinal extracts then treated the collagenolysis-induction agents. Pretreatment of the extracts for 1 h, which by itself had little effect on cell survival, did not enhance

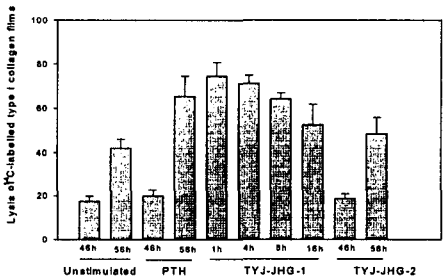


Fig. 4.

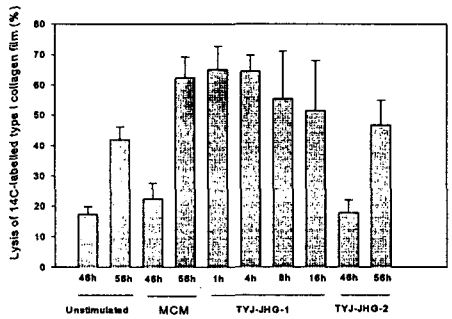


Fig. 5.

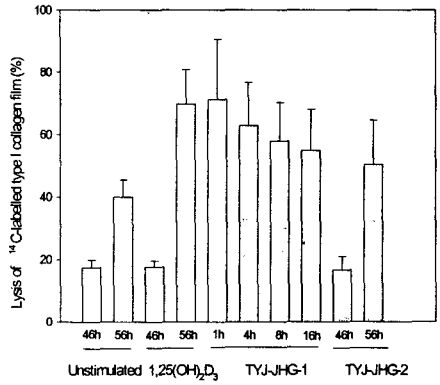


Fig. 6.

Fig.4~6. Lysis of ^{14}C-labelled type I collagen films by mouse calvarial osteoblasts and effects of T.Y.J.-J.H.G. extracts on PTH, 1,25(OH)$_2$D$_3$ and MCM -induced collagenolysis.

T.Y.J-J.H.G.-1 , The cells were treated with PTH, 1,25(OH)$_2$D$_3$ and MCM , and then
after 56hrs, T.Y.J.-J.H.G. extracts were further treated to the cells, then PTH induced
collagenolysis were assayed with time courses of 1, 4, 8 and 16 hrs.
T.Y.J-J.H.G.-2 , First, the cells were treated with T.Y.J.-J.H.G. extracts for 1 hr, and
then the cells were further treated with PTH and after 46 and 56 hrs, PTH-induced
collagenolysis were assayed to see T.Y.J.-J.H.G. -pretreatment effect.

the collagenolysis, nor significantly reduced the collagenolysis by pretreatment (Fig. 4-6).

3. Effects of oriental medicinal extracts such as T.Y.J.-J.H.G. on IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells.

Since interleikin-1(IL-1) is a major constituent of MCM, we tested the ability of recombinant human IL-1 (rhIL-1α; 0.1-20.0 ng/ml) to stimulate collagen degradation by the cells; maximal collagenolysis (69%) was again only achieved after 56 h with the optima dose of 10 ng/ml (Fig. 7). Also, IL-1βwas tested for stimulation of collagen degradation (0.1 - 2.0 ng/ml). The maximal collagenolysis was obtained after 56 h with the optima dose of 5 ng/ml (Fig. 8). Therefore, it was suggested that M CM was included as a bone resorptive agent because target cells are likely to be exposed in vivo to mixtures rather than individual cytokines, particularly during inflammation.

To examine the anti-collagenolysis of the medicinal extracts of T.Y.J.-J.H.G. on IL-1α and IL-1β-induced collagenolysis in calvarial osteoblast cells, various concentrations of the T.Y.J.-J.H.G. extracts were tested for whether they could protect against rhIL-1α (2 ng/ml) or IL-1β(1 ng/ml)-induced collagenolysis in the mouse calvarial cells (Fig. 7 and 8). Cell viability was not significantly affected by treatment with the indicated concentration of the extracts alone, as examined also in MT T assays.

Furthermore, the T.Y.J.-J.H.G. extracts 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 [3], although there are some controversies. Thus, we examined the effect of the pretreatment with a various concentrations of the T.Y.J.-J.H.G. extracts then treated the colla-

genolysis-induction agents. Pretreatment of the T.Y.J.-J.H.G.extracts for 1 h, which by itself had little effect on cell survival, did not enhance the collagenolysis, nor significantly reduced the collagenolysis by pretreatment (Fig. 7,8).

4. Determination of gelatin-degrading activities of mouse osteoblasts and effects of the medicinal extracts of T.Y.J.-J.H.G. on PTH (2 units/ml), or MCM (5%, v/v), or 1,25(OH)$_2$D$_3$ (10 ng/ml), IL-1α and IL-1β-induced gelatin-degrading activities of mouse osteoblasts.

To assess the gelatin-degrading activities of mouse osteoblast conditioned media culture supernatants were electrophoresed on SDS -polyacrylamide gels containing gelatin under non-reducing conditions. These demonstrated that mouse osteoblasts constitutively synthesized progelatinase-A irrespective of the substratum (Fig. 9, lane 1-2). When the cells were cultured on type I collagen films, however, the 62 kDa active species was generated from the inactive 66kDa proform(under non -reducing conditions 72 kDa gelatinase runs at the slightly lower Mr of 66 kDa), with further processing to a 40 kDa via 42 kDa form following PTH (2 units/ml)(Fig. 10, lane 1), or 1,25(OH)$_2$D$_3$ (10 ng/ml)(Fig. 10, lane 2), or IL-1β (Fig. 10, lane 1), and IL-1α

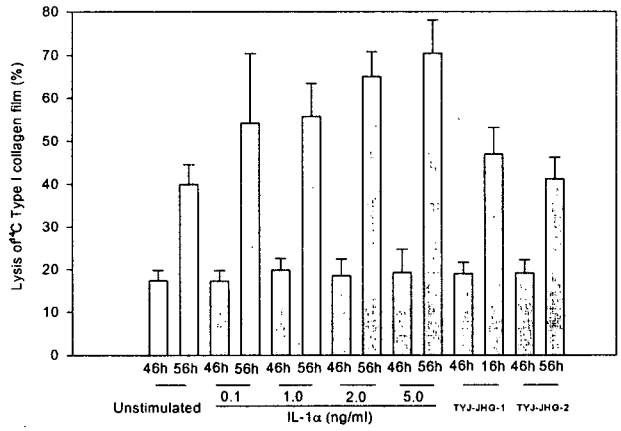


Fig. 7. Effect of T.Y.J.-J.H.G. extracts on IL-1α(2.0 ng/ml)-induced collagenolysis by mouse calvarial osteoblasts

T.Y.J-J.H.G.-1 , The cells were treated with IL-1α(2,0ng/ml), and then after 56 hrs, T.Y.J.-J.H.G. extracts were further treated to the cells, then IL-1α(2,0ng/ml) induced collagenolysis were assayed with time courses of 1 and 16 hrs.
T.Y.J-J.H.G.-2 , First, the cells were treated with T.Y.J.-J.H.G. extracts for 1 hr, and then the cells were further treated with IL-1α(2.ng/ml) and after 46 and 56 hrs, IL-1α(2,0ng/ml)-induced collagenolysis were assayed to see T.Y.J.-J.H.G. pretreatment effect.

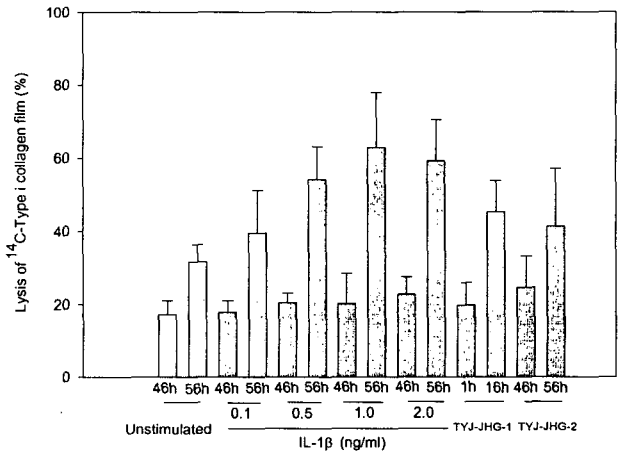


Fig. 8. Effect of IL-1β (0.1-2.0 ng/ml) on lysis of ^{14}C-labelled type I collagen films by mouse calvarial osteoblasts and effects of T.Y.J.-J.H.G. extracts on IL-1β (1.0 ng/ml)-induced collagenolysis.

(data not shown). However, in a case of MC M treatment, MCM (5%, v/v) stimulation showed a little effect of gelatinase processing (data not shown). These agents were alone in being able to stimulate the progelatinase-A. These findings are similar to recent reports and suggest an important role for the extracellular matrix in the activation of progelatinases[4,6,12]. The precise mechanism is unknown but the process appears to be specific to type I collagen.

Interestingly, the T.Y.J.-J.H.G. extracts were shown to have the inhibiting effects against gelatinase enzyme and prosseing activity induced by the bone resortion agents of PTH (Fig. 10, lane 4), 1,25(OH)$_2$D$_3$ (Fig. 10, lane 5), IL-1β (Fig. 10, lane 6) and IL-1α (data not shown). Their effects were highly effective to inhibit the gelatinase production and processing. When we examined the effect of the pretreatment with a various concentrations of the T.Y.J.-J.H.G. extracts then treated the gelatinase-induction agents. Pretrea-

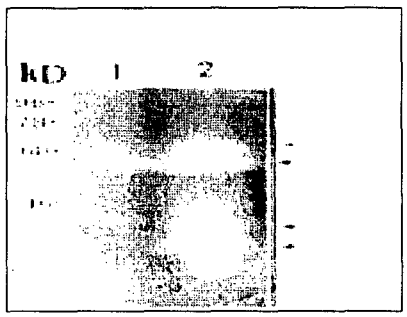


Fig. 9. Detection of mouse gelatinase-A (72 kDa) by zymography. and effects of medicinal extracts on IL-1β (1.0 ng/ml)-induced collagenolysis.

The effects of osteotropic agents are shown: unstimulated, lane 1; MCM, lane 2. Arrows indicate active gelatinase A(66 kDa) and the new enzyme forms (42 and 40 kDa). Arrowhead indicates progelatinase.

tment of the extracts for 1 h, which by itself had little effect on cell survival, did not enhance the collagenolysis, nor significantly reduced the collagenolysis by pretreatment (data not shown).

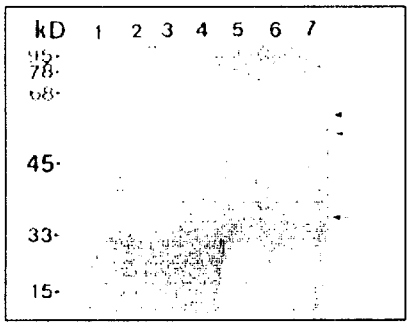


Fig. 10. Detection of mouse gelatinase-A (72 kDa) by zymography.

The effects of osteotropic agents are shown: unstimulated, lane 1; MCM, lane 2. Arrows indicate active gelatinase A(66 kDa) and the new enzyme forms (42 and 40 kDa). Arrowhead indicates progelatinase. T.Y.J-J.H.G.-treated sample: lane 4, PTH; lane 5, $1,25(OH)_2D_3$; lane 6, IL-1β. Bone resorption agent-treated sample: Lane 1, PTH; lane 2, $1,25(OH)_2D_3$; lane 3, IL-1β.

5. Bone resorption activity of IL-1 and effects of T.Y.J.-J.H.G. extracts on IL-1α- and IL-1β-induced bone resorption mouse calvarial osteoblasts.

It is well known in cellular and molecular aspects that IL-1α- and IL-1β have similar dose dependent responses in most biological systems[12]. However, the dose response for stimulating bone resorption differed significantly in the fetal mouse long bone organ tissue culture, as shown in Fig. 11. Human IL-1β is approximately 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 ($P<0.01$) 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[13]. It was known that IL-1α- and IL-1β generally have the same potency and biological activity and bind to the same receptor[13]. 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. T.Y.J.-J.H.G. extracts were shown to have the inhibiting effects against IL-1α- and IL-1β-stimulated bone resorption and the effect of the pretreatment with a various concentrations of the medicinal extracts were significant (Fig. 11, 12).

6. Effects of nonsteroidal anti-inflammatory agents of indomethacin and dexamethasone on the dose dependent responses of IL-1α- and IL-1β.

When it was examined the effects of indomethacin and dexamethasone on the dose dependent responses of IL-1α (Fig. 13) and IL-1β (Fi.g. 14). Indomethacin produced a rightward shift in the IL-1 dose response curve. Analysis of covariance indicated no significant difference in the slopes of the

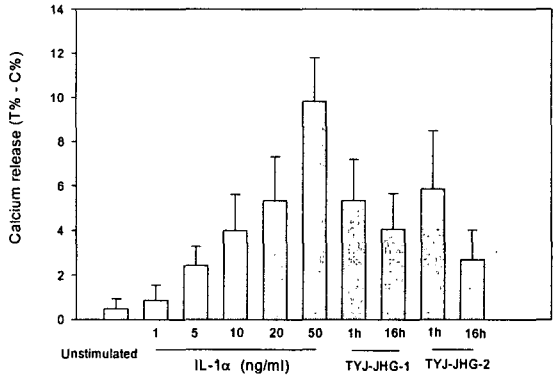


Fig. 11. Dose-dependent responses for IL-1α-stimulated bone resorption and effects of T.Y.J.-J.H.G. extracts on IL-1α-induced bone resorption.

T.Y.J.-J.H.G-1 , The cells were treated with IL-1α(50ng/ml) and after 6 hrs. T.Y.J.-J.H.G. extracts were further treated to the cells. And then IL-1 α stimulated bone resorption were assayed with time courses of 1 and 16 hrs.
T.Y.J.-J.H.G-2 , First, the cells were treated with T.Y.J.-J.H.G extract for 1 hr. and then the cells were further treated with IL-1α (50ng/ml), and after 1 and 16 hr, IL-1α stimulated bone resorption were assayed to see T.Y.J.-J.H.G pretreatment effect.

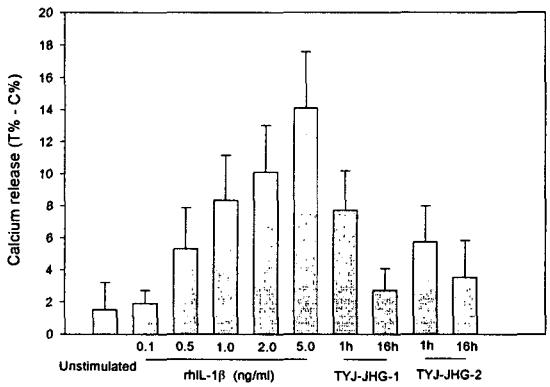
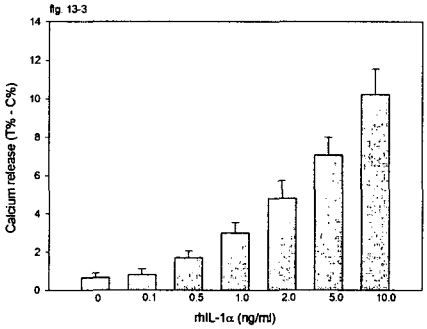


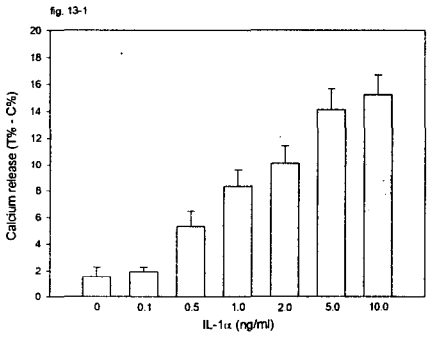
Fig. 12. Dose-dependent responses for IL-1β-stimulated bone resorption and effects of T.Y.J.-J.H.G. extracts on IL-1β-induced bone resorption.

two curves. Variance ratio test showed a hi-ghly significant difference ($P<0.01$) between the adjusted (for IL-1α or IL-1β level) means of the groups with and without indomethacin and dexamethasone. However, these prostaglandin synthase inhibitors of indomethacin and dexomathasone did not inhibited IL-1-stimulated bone resorption in the mouse calvarial tissue culture system (data not shown), but inhibited only in the fetal mouse long bone organ tissue culture.

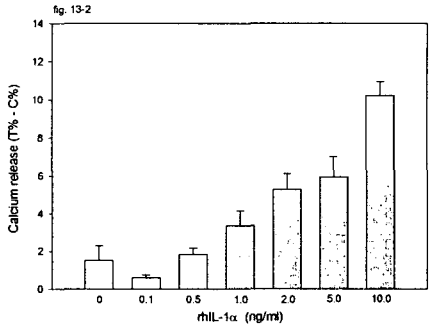


(c)

Fig. 13. Effects of indomethacin and dexamethasone on the dose-dependent response of IL-1α. (A) IL-1α alone, (B) rhIL-1α in the presence of 1uM indomethacin. (C) rhIL-1α in the presence of 1uM dexametasone in treatment and control bone cultures.



(a)



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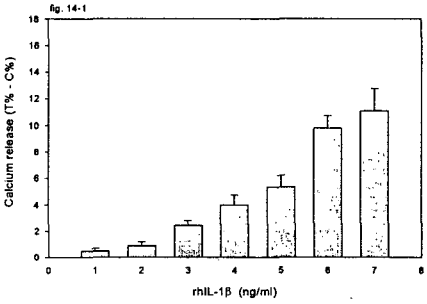

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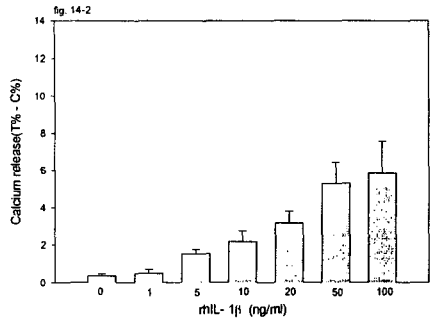

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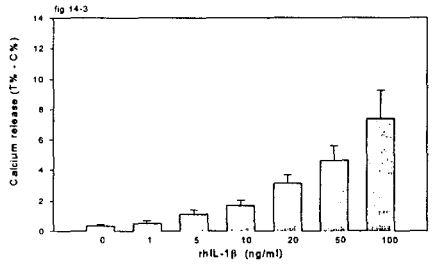

(c)

Fig. 14. Effects of indomethacin and dexametasone on the dose-dependent response of IL-1β.

(A) IL-1β alone, (B) rhIL-1β in the presence of 1uM indomethacin. (C) rhIL-1β in the presence of 1uM dexametasone in treatment and control bone cultures.

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