대한한방부인과학회지
THE JOURNAL OF ORIENTAL OBSTETRICS & GYNECOLOGY
VOL.20 NO.4 : 056-073 (2007)

자하거의 tyrosine kinase Src, cyclooxygenase 발현, PGE2 합성 등의 저해를 통한 골질재흡수 억제효과

*동국대학교 한의과대학 부인과학교실, **이태균한의원
양정민*, 이태균**, 김동일*

ABSTRACT

Jahage, *Hominis Placenta*(HP), suppress bone resorption by inhibition of tyrosine kinase Src, cyclooxygenase expression and PGE2 synthesis

Jeong-Min Yang*, Tae-Kyun Lee**, Dong-Il Kim*
*Dept. of OB & GY, College of Oriental Medicine, Dongguk University
**Dr. Lee's Traditional Korean Medical Clinic

Purpose: 이 실험은 골다공증의 치료약물로 자하거의 골질재흡수 억제효과를 검토하기 위하여 설계되었다.

Methods: 자하거의 골질재흡수 효과를 확인하기 위하여 생쥐의 두개골 골모세포를 이용하여 Cyclooxygenase-1(COX-1), COX-2, TGF-β, L-1β, TNF-α, IL-6, prostaglandin E2등의 활성화 정도를 측정하였으며, 골조직의 미세구조적 변화를 확인하였다.

Results: 자하거는 IL-1β, TNF-α, IL-6 또는 그 세가지의 조합에 의하여 유발된 PGE2의 생성 뿐만 아니라 COX-2 mRNA 수치도 감소시켰으나 COX-1 mRNA 수치에는 영향을 주지 않았다. 이로써 자하거는 시험관내에서 그리고 생체내에서 펩티드의 인산화를 억제함으로써 골의 재흡수를 저해하였다. 그리고 자하거는 생쥐에서 IL-1β에 의해 유발된 고칼슘혈증을 감소시켰고, 골의 재흡수를 저해하는 경로를 통하여 골에 대한 보호효과를 보여줌으로써 조기에 난소 절제한 쥐에서 골질감소와 미세구조적 변화를 부분적으로 방지하였다. 이러한 결과는 PGE2 생성에 대한 IL-1β, TNF-α, IL-6사이의 상승효과는 COX-2의 유전자 발현이 증가한 결과이며 이러한 tyrosine kinase가 생쥐의 두개골 골모세포에서 COX-2의 신호전달에 관계한다는 것을 보여준다.

Conclusion: 자하거가 생쥐의 두개골 골모세포에서 여러 신호전달물질의 활성화를 통하여 골질재흡수를 저해하는 특성을 확인함으로써 앞으로 골다공증의 예방과 치료에 대한 추가적인 임상연구가 필요할 것으로 사료된다.

Key Words: Osteoblast, Osteoporosis, Protein tyrosine kinase inhibitor, Jahage, *Hominis Placenta*(HP)

교신저자(김동일) : 경기도 고양시 일산동구 식사동 814 동국대일산한방병원 한방여성학과
전화 : 031-961-9062 이메일 : obgykdi@hanmail.net

Ⅰ. Introduction

It is well known that Korean herbal medicine, Jahage (*Hominis Placenta) (HP)* is effective for the treatment of inflammation, arteriosclerosis, and gynecological diseases such as osteophoresis and bone resorption according to the ancient Chinese and Korean medicinal and herbal literature[1]. The HP is also known as an effective biological response modifier for augmenting host homeostasis of body circulation[1]. The pharmacological action of HP has been limitedly studied in regard to gynecological diseases. This herbal medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects[2]. In addition, it has anti-inflammatory properties. It is used in the treatment of Yin deficiency of liver and kidney, hectic fever, night sweat, and dizziness[3]. Thus, it still occupies an important place in traditional Oriental medicine.

Natural products of plant origin are still a major part of traditional medicinal systems in developing countries. There is also a resurgence of interest in herbal medicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[3,4]. A literature survey on the fetal human *Hominis Placenta* revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. The need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

The cytokine interleukin-1β(IL-1β), interleukin-6(IL-6) and tumor necrosis factor-α(TNF-α), which are produced mainly by actibated monocytes or macrophages, stimulate bone resorption as well as enhance PGE_2 production in several type of cells including calvarial osteoblasts[5-8]. Prostaglandins play an important role in the inflammatory processes including periodontitis, osteoporosis and RA. The production of prostaglandins is regulated by the rate-limiting enzymes phospholipase A_2(PLA_2) and cyclooxygenase(COX). The PLA_2 enzymes catalyzes the liberation of arachidonic acid(AA) from membrane phospholipids whereas the enzyme COX mediates conversion of AA to prostaglandins[8]. The enzyme COX exists at least in two isoforms, the constitutive COX and the inducible COX-2 [9-11].

Osteoporosis is a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk. The low bone mass results from an imbalance between bone formation and bone resorption, coupled processes that maintain skeletal integrity. Most conditions that lead to osteoporosis

(estrogen deficiency in postmenopausal women, hyperparathyroidism, hyperthyroidism, and corticosteroid treatment) are associated with increased bone resorption, which is determined by the number and activity of bone-resorbing cells, or osteoclasts[12]. The differentiated osteoclast is a large, multinucleated, highly motile cell of hematopoietic origin[13].

Soriano *et al.*[14] demonstrated that mutant mice with a disrupted *Src* gene develop osteopetrosis, a disease characterized by a lack of osteoclastic activity. These Src-deficient mice had normal numbers of osteoclasts, but failed to polarize and form ruffled borders, indicating that Src isnot required for osteoclast formation but, rather, for their function[14-16]. These findings are consistent with the high expression of Src in osteoclasts, comparable to that in brain and platelets[16-18]. The exact nature of the role played by Src is not clear since osteoclastic bone resorption is a complex and incompletely understood process. The tyrosine kinase Src has been implicated in the process of osteoclast-mediated bone resorption. It has been shown in different cell types that phosphorylation by Src, and related kinases, of proteins proposed to participate or regulate the cytoskeletal architecture is one important requirement for their proper function[19]. It was reported that phosphorylation of Cbl is Src dependent in osteoclasts and that Cbl antisense oligonucleotides can block osteoclastic bone resorption *in vitro*[20]. Another possible downstream Src target, phosphatidylinositol 3-kinase, has been implicated in intracellular membrane trafficking and shown to be located at the osteoclast ruffled border, and is required for osteoclast-mediated bone resorption[21].

The demonstration that mutant mice with a disrupted *Src*gene have functionally inactive osteoclasts, in the absence of any other overt pathological signs, is clear evidence that Src plays a unique and crucial role in osteoclastic bone resorption. However, it has not yet been unequivocally shown whether the observed bone effect is due to the lack of kinase activity or to the absence of a functional SH2 domain, or indeed to another mechanism. It is unclear whether integrin-mediated signaling plays any role in those functions affected by the lack of Src in osteoclasts. Only when a potent, specific Src inhibitor with an acceptable pharmacokinetic profile is available this point can be resolved in experiments with ovariectomized rats.

To examine the inhibitory effect of some oriental medicinal extracts on the bone resorption and collagenolysis in the mouse calvarial bone cells, we have screened and assayed the inhibitory activities of HP. Although the effectiveness of HP for gynecological

and inflammatory diseases has been widely demonstrated by clinical administration, the scientific and acting mechanisms for those are not understood and elucidated. It is generally known that inflammation induces bone resorption and osteophoresis. Therefore, anti-bone resorption activity may be assessed by the effect on osteoblastic cells. The present paper reports the effect of extracts obtained from HP on cytokine-induced experimental bone resorption in mouse calvarial cells. Thus, it was concluded that the HP extracts are highly stable and applicable to clinical uses in osteoporosis.

II. Materials and methods

1. Medicinal herbal formulation

The aqueous extracts of HP, which was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University College of Oriental Medicine (Kyungju, Korea).

2. Reagents

Radiochemicals were from Amersham International Co. (Seoul, Korea). All other chemicals and biochemicals were of analytical grade and were purchased from Sigma Chem. Co. (St. Louis, MO) or Boehringer Mannheim Biochemicals (Seoul, Korea).

Human recombinant IL-1 was our deposit[22]. TNF-α and IL-6 were purchased from R&D Systems (Funakoshi, Co., Ltd., Tokyo, Japan). Herbimycin A was from Sigma Chemical CO. (St. Louis, MO, USA). Radioimmunoassay kits for PGE_2 were from Ammersham. It was stored in aliquots of 100 mg dissolved in 91 mL of 100 mmol/L Tris buffer (pH 7.8) containing 2 mmol/L sodium azide, and diluted with phosphate-buffered saline (PBS) prior to use.

3. Calvarial osteoblast cultures

Cultures of osteoblast cells were established from calvaria obtained from healthy 10 mice with 2 months of age. Minced pieces of the tissue were explanted to 25 cm2 Falcon tissue culture flasks containing 5 ml of Eagle's basal medium (BME). The osteoblasts were obtained by trypsinisation of the primary outgrowth of cells as previously described[23]. Osteoblasts were seeded and grown in BME supplemented with 5% fetal calf serum, L-glutamine, penicillin-streptomycin and HEPES for 24 h. The cell layers were then rinsed three times with serum-free BME medium and incubated in the absence or presence of IL-1β, TBF-a, IL-6 and HP alone or in combinations at the concentrations indicated in the legends to figures and tables. At the end of the incubation period, indicated in the legends to figures and tables, the monolayer was frozen for isolation of total RNA. The cells used for the

experiments proliferated in the logarithmic phase between the 6th and the 12th passage.

4. Quantification of mRNA for COX-1 and COX-2.

The cells were scraped in 2 ml of 10 mM EDTA (pH8.0), 0.5% SDS and RNA was isoalted according to Sambrook et al.[24]. The levels of mRNA for COX-1 and COX-2 were determined by southern hybridization which was carried out by using RNA probes, synthesized *in vitro* using a RNA trasncription kit, and labeled with 35S-rCTP[24].

5. Prostaglandin E_2 determination and Protein kinase assays

The amount of PGE_2 was determined in the medium by using commercially avaiable radioimmunoassay kits with [^{125}I]-PGE_2 antiserum. The PGE_2 antiserum has 30% cross-reactivity with in PGE_2.

32P-based tyrosine kinase assays with human Src enzyme [Upstate Biotechnology, Inc. (UBI) Lake Placid, NY] were performed with a kinase buffer containing 20 mmol/L Tris, pH 7.4, 10 mmol/L $MgCl_2$, 0.1 mmol/L sodium vanadate, and 1 mmol/L dithiothreitol (DTT), substrates poly Glu-Tyr (4:1), or optimal Src substrate (OSS)[25] peptide at 1 and 0.5 mg/mL; 50 umol/L adenosine triphosphate (ATP), and 0.5-2 μCi of 33P-rATP per assay. Incubations were performed for 15 min at room temperature. The reactions were stopped with 33 mmol/L ethylenediaminetetraacetic acid (EDTA) and spotted onto phosphocellulose paper squares (Whatman P81, Maidstone, England). The paper squares were washed three times with 0.5% phosphoric acid, once with absolute ethanol, and air-dried, and the paper-bound radioactivity was quantified in a liquid scintillation counter. Chicken Src was expressed and purified as described before[26]; the assay was similar to that with human Src except that 6 umol/L ATP was used for routine screening, and the phosphorylated poly Glu-Tyr was retained on an Immobilon-P membrane (Millipore, Volketswil, Switzerland).

6. IC_{50} calculation

The IC_{50} values were determined graphically as the concentration of inhibitor which reduced the signal of untreated control cells by 50%. The exposures that had an experimental 50% control value (equals the signal of one half of the protein analyzed) approaching the calculated 50% signal (equals calculated half of the control value) were taken as being in the linear range for the enhanced chemiluminescence (ECL) reaction and X-ray film response.

7. Immunoprecipitation and immunodepletion

Cleared cellular lysates were diluted with NP-40-lysis buffer to a final protein concentration of 1 mg/mL and incubated with 2-4 ㎍ of appropriate antibody for 2-4 h on ice on an orbital shaker. The immune complexes were subsequently captured with 10 ㎕ of protein A- or protein G-Sepharose for 30 min. The beads were washed according to one of the following protocols: (a) twice with 1 L of TNEN buffer (50 mmol/L Tris HCl, pH 8.0, 500 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP-40), twice with 1 ㎖ of TNE (TNEN without NP-40), and once with 30 mmol/L Tris, pH 7.5; or (b) four times with 1 ㎖ of lysis buffer (the second two washes without protease inhibitors) and once with 50 mmol/L Tris, pH 7.4. The proteins in immune complexes were analyzed either by SDS-PAGE Western blotting or in immune complex kinase assays. For Western blotting analysis, the antigens were released from the beads by boiling in 50 ㎕ SDS sample buffer, proteins were resolved by SDS-PAGE, and immunoblotting was done as described above. Src immune complexes were analyzed on longer gels (8 × 9 cm; Mighty Minigel; Pharmacia Biotechnologies, Dubendorf, Switzerland) at 20 mA/gel to achieve separation or Src and antibody chains. Gels were dried and exposed on a PhosphorImager screen for 4-40 h. The bands of interest were quantified with the ImageQuant program (Molecular Dynamics) and corrected for the background signal. The protein in each fraction was determined as previously described, and equal amounts of protein (7 ㎍) from the lysate and immunodepleted supernatants were subjected to SDS-PAGE.

8. *In vitro* bone resorption assay

Fetal rat long bones were prepared and cultured as described by Feyen *et al.*[27]. In brief, timed pregnant Sprague-Dawley rats were injected with radiolabeled 45Ca subcutaneously (s.c.) (100 µCi) on the 18th day of gestation. The following day, radii and ulnae were dissected and then precultured in 0.5 mL of BGJ medium supplemented with 1 mg/mL of bovine serum albumin (BSA) in 24 well tissue culture plates in a CO_2 incubator at 37℃ for 24 h. The bone explants were then cultured in the presence or absence of the agents to be tested for 2 days. The medium was removed and replaced with fresh medium supplemented with the test agents, and culture was continued for another 3 days before terminating the experiment. Aliquots of conditioned medium of day 2 and day 5 and the acid extract [trichloroacetic acid (TCA), 5% (w/v)] of the bone explants were counted for 45Ca by liquid scintillation. Bone resorption was assessed as the percentage of total 45Ca that was released into the medium.

9. IL-1β-induced hypercalcemia in mice

The method of Sabatini *et al.*[28] was used to induce hypercalcemia in mice. An Alzet minipump (type 1003D; Alza Corporation, Palo Alto, CA) was implanted on the upper side of the neck of each mouse while in anesthesia with halothane/air (4:96%). The minipump infused IL-1b dissolved in PBS at a rate of 2 mg/day for 72 h. The control group received a similar minipump infusion of PBS. The test compounds were administered subcutaneously twice daily at 6:30 and 2:30. The last administration (seventh injection) was given in the morning of day 4; 2 h thereafter the mice were killed and blood samples were collected. The total serum calcium concentration was determined colorimetrically at 570 nm with *o*-cresolphthalein complexone using a commercially available kit (MPR Calcium; Boehringer Mannheim, Germany) in a microtitre plate format. The serum amyloid protein concentration of the blood samples was determined by an ELISA method[29].

10. Bone loss in ovariectomized rats

Four groups of eight rats and mouse were ovariectomized (ovx) by electrotomy via the dorsal route in anesthesia. One additional group of rats served as the intact control group. Immediately after ovariectomy, two ovx groups were given 10 and 50 mg/kg of HP by oral gavage twice daily on weekdays and once a day at weekends for 6 weeks. The intact control group and one ovx control group were similarly treated with 0.9% (w/v) saline. The first lumbar vertebra (LV-1) and both femurs were removed for biochemical and morphometric analyses. Calcium and hydroxyproline in femoral trabecular bone were determined by chemical analysis according to the method of Gunness-Hey and Hock[30]. Briefly, the femurs were cut in half at the middiaphysis and the proximal halves were discarded. The epiphysis of the distal half was cut off and the bone was split into saggital halves, then the marrow was flushed out with water. With a dental curette, the trabecular bone was scraped out of both cortical shells, combined and put into 5% trichloroacetic acid (TCA). After standing 16 h at room temperature, the TCA extract was removed and its calcium content determined colorimetrically with *o*-cresolphthalein complexone. The remaining demineralized matrix was successively washed with ethanol and methylenechloride and dried under vacuum prior to determining its hydroxyproline content. The dried matrix was hydrolyzed with 6 mol/L HCl at 120℃for 5 h and the hydroxyproline content was determined colorimetrically[31].

11. Statistics

Results of the above animal studies are given as mean ±standard error of

the mean (SEM). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) and by Tukey's multiple comparison test against the control group.

Ⅲ. Results

1. Synergistic Inhibitory effect of HP and TGF-β on TNF-α, IL-1β and IL-6 -induced COX mRNA levels and PGE_2 production

The cytokine TGF-β (10 U/ml) significantly reduced COX-2 mRNA levels in cultures of osteoblasts (Fig. 1 A, B, C) when TGF-β were treated after TNF-α, IL-βand IL-6 treatment. When 100 μg/ml of HP was treated with TGF-β(10 U/ml) to the osteoblast cells to examine whether the HP inhibits synergistically the COX mRNA levels and PGE_2 production after TNF-α, IL-β and IL-6 treatment, COX-2 mRNA expression and PGE_2 production were greatly reduced by HP(100 μg/ml) synergistically with a significant difference (P<0.001). This result also indicated that the HP is acting in stage of COX-2 mRNA transcription and PGE_2 synthesis of the osteoblast cells (Fig. 1A, B, C).

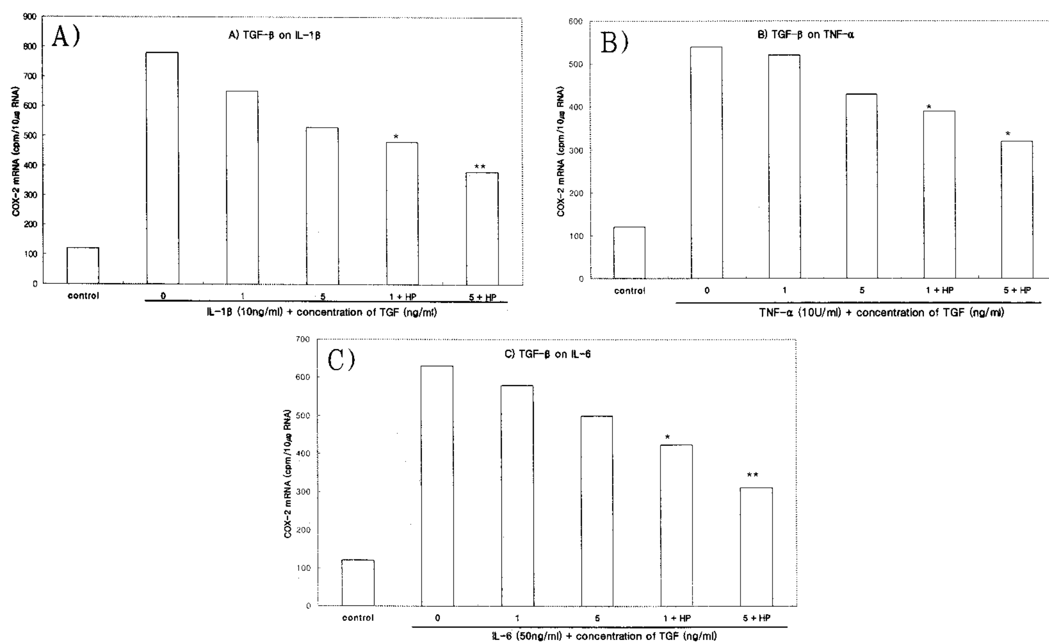

Fig. 1. Synergistic inhibitory activity of HP (100μg/ml) on inhibitory effect of TGF-β on IL-1β-induced COX mRNA levels.
Effect of TGF-b, at different concentrations, in the absence or presence of IL-1B (10 ng/ml), in 6 h cultures, was assayed to see the expression of COX-2 mRNA level in mouse osteoblasts (N=6). Mean+-SD of triplicate experiments representing one of three separate experiments with similar results. * significantly different from untreated control cells (P<0.01), **, significantly different (P<0.001).

2. Inhibitory activity of HP on effect of TNF-α, IL-1βand IL-6 alone or in combination on PGE_2 production

The cytokine IL-1β and to a lesser extent IL-6 (≥10 ng/ml), stimulated PGE_2 production in osteoblasts (Fig. 2A,B). Furthermore, the increase of COX-2 mRNA levels stimulated by the combination of IL-1β and IL-6 were accompanied by a synergistic increase of PGE_2 production which was dependent on the concentration of IL-6. HP treatment with 100ng/ml of IL-6 in the presence or absence of IL-1β (Fig. 2A) and TNF-α (Fig. 2B) showed the significant inhibition of PGE_2 production which was dependent on the concentration of IL-6.

Neither IL-1β, TNF-α, and IL-6 not the combination of the three cytokines increased COX-1 mRNA levels in mouse osteoblasts(Fig. 3). Furthermore, when TNF-α or IL-6 in the absence or presence of HP (100 ㎍/ml) in 6 h of cultures, on COX-1 and COX-2 mRNA level were assayed in osteoblasts, the COX-2 mRNA levels were significantly decreased.

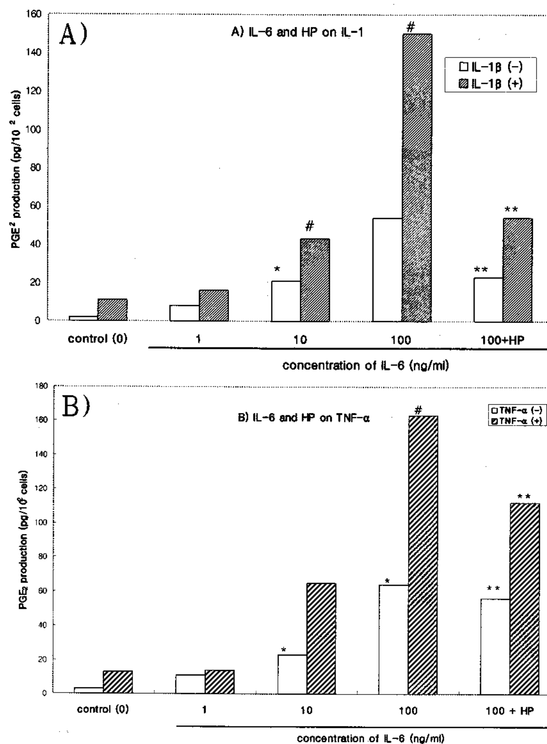

Fig. 2. Inhibitory activity of HP (100+ug/ml) of effect of IL-6, at different concentrations,in the absence or presence of IL-1β (10 ng/ml) or TNF-α, in 6 h cultures, on the PGE_2 production in mouse osteoblasts (N=6).
Mean+-SD of triplicate experiments representing one of three separate experiments with similar results. * significantly differentfrom untreated control cells (P<0.01), #, significantly different from IL-1βor IL-6-treated cells (P<0.001). **, significantly different from HP and 100ng/ml IL-6-treated cells (P<0.001).

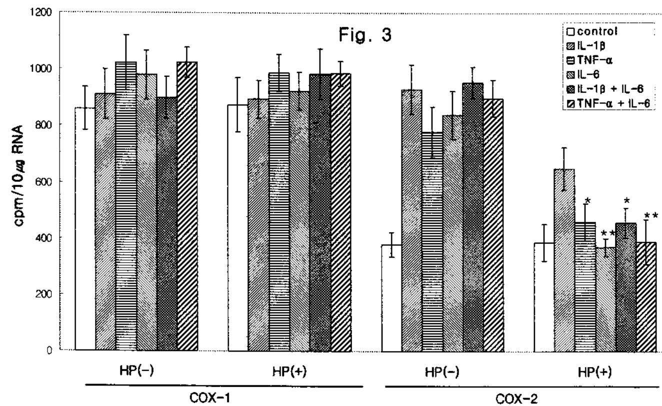

Fig. 3. Effect of IL-1β, TNF-α or IL-6 in the absence or presence of HP (100 ug/ml) in 6 h of cultures, on COX-1 and COX-2 mRNA level in osteoblasts (N=6).

3. Inhibitory effect of HP and TGF-β on TNF-α, IL-1β and IL-6-induced PGE2 production

The cytokine TGF-β (10 U/ml) significantly reduced PGE_2 production levels in cultures of osteoblasts (Fig. 4A,B,C) when TGF-β were treated after TNF-α, IL-1β and IL-6 treatment. Inhibitory effect of TGF-β and a combination of TGF-β plus(+) HP on IL-1β-induced PGE_2 production were examined. Effect of TGF-β, at different concentrations, in the absence or presence of IL-1β (10 ng/ml), in 6 h cultures, was assayed to see the PGE_2 production level in mouse osteoblasts (N=6). As shown in Fig. 4 A,B,C, TNF-α, IL-1βand IL-6 -induced PGE_2 production were significantly decreased ($P<0.001$) by 5 or 10ng/ml TGF-β, and more stronlgy by TGF-β+ HP.

4. Effect of HP and tyrosine kinase inhibitors on COX-2 mRNA level and PGE_2 production in mouse calvarial osteoblasts

To compare the biological activity of HP ,the effects of tyrosine kinase inhibitors, herbimycin A (Herb), on COX-2 mRNA level and PGE_2 production was also investigated (Fig. 5). The specific tyrosine kinase inhibitor, HERB and HP strongly reduced COX-2 mRNA levels induced by IL-1B, TNF-a or IL-6 or the combination of them. However, HERB and HP did not affect COX-1 mRNA level induced by the cytokines (data not shown). Also, the specific tyrosine kinase inhibitor, HERB, did not affect COX-1 mRNA level in osteoblasts (Fig. 5). These indicate that HP plays a role in inhibiting tyrosine kinase activities of the bone resorbing cells.

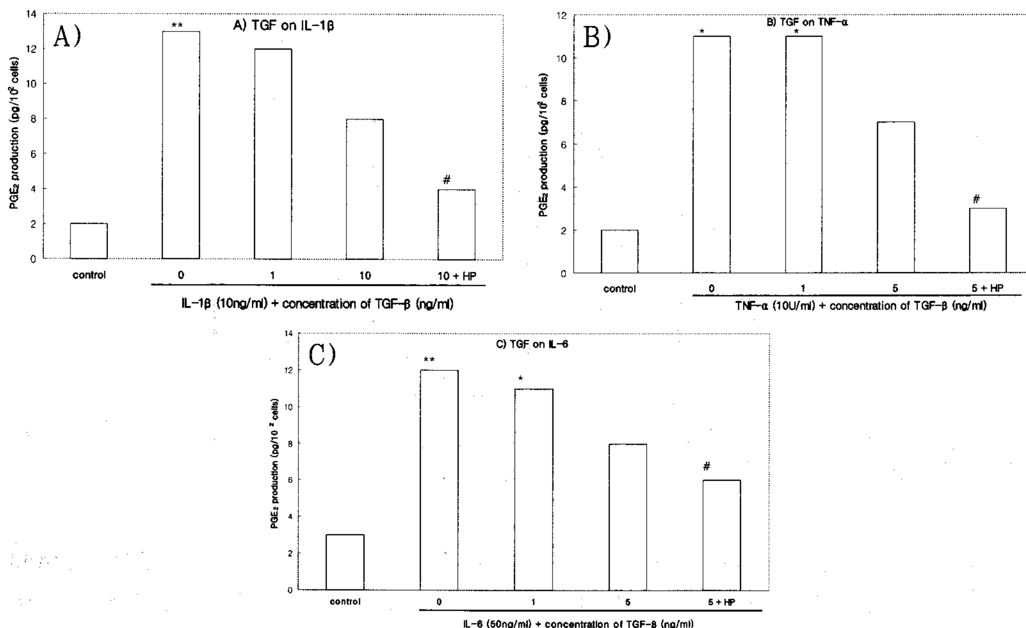

Fig. 4. Inhibitory effect of TGF-β and a combination of TGF-β plus(+) HP on IL-1β-induced PGE_2 production

Effect of TGF-β, at different concentrations, in the absence or presence of IL-1β (10 ng/ml), in 6 h cultures, was assayed to see the PGE_2 production level in mouse osteoblasts (N=6). Mean±SD of triplicate experiments representing one of three separate experiments with similar results. * significantly different from untreated control cells ($P<0.01$), **, significantly different ($P<0.001$). # significant different from 5 or 10ng/ml TGF-β and TGF-β+ HP

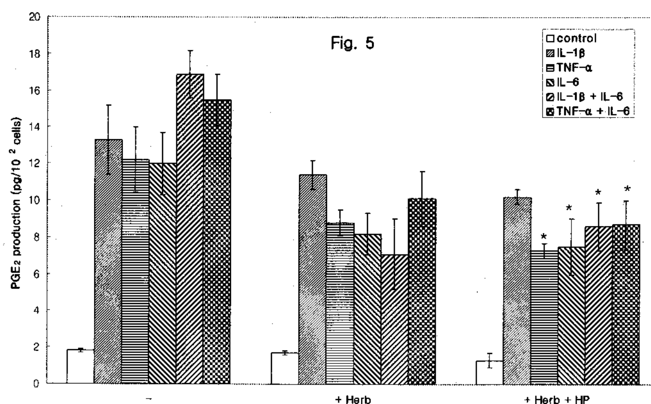

Fig. 5. Effect of IL-1β, TNF-αor IL-6 in the absence or presence of HERB (1.0 uM) and HP in 6 h of cultures, on PGE_2 production in osteoblasts (N=6).

5. *In vitro* activity of HP on Src inhibition by means of IC_{50} value

HP has been identified as potent inhibitors of Src in enzymatic assays. The finding that HP exhibit potent tyrosine kinase inhibition is novel. Fig. 6 shows *in vitro* activity of HP. The HP compound inhibited chicken Src

with an IC_{50} value of 0.1 umol/L, was selective against the serine/threonine kinase Cdc2. In conclusion, HP increased the potency of Src inhibition *in vitro* and chose HP for further characterization in vitro and *in vivo*. Furthermore, inhibitory IC_{50} values of HP (㎍/ml for HP) were calculated for the inhibition of purified protein kinase activity in enzymatic assays. When chicken recombinant Src enzyme was used in these assays, HP inhibited chicken Src with IC_{50} value of 16 ㎍/ml.

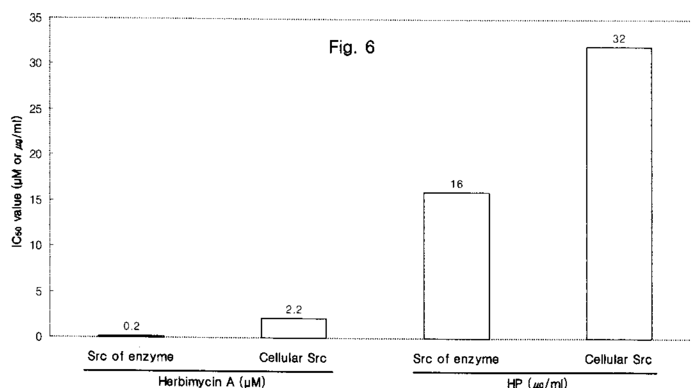

Fig. 6. Potency of inhibitor, HERB and HP toward protein kinase *in vitro*.
IC_{50} values (umol/L for HERB or ug/ml for HP) were calculated for the inhibition of purified protein kinase activity in enzymatic assays. Chicken recombinant Src enzyme was used in these assays. For a descriptionof enzymes and assays, see the *Materials and Methods* section.

6. HP inhibits PTH-induced bone resorption *in vitro*

The effect of the Src inhibitor HP on bone resorption was evaluated *in vitro* in the fetal rat long bone organ culture system described previously[27]. Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH-(1-34) in the presence or absence of HP over the concentration range 0.1-100 ug/ml. Bone resorption was assessed as the percentage release of 45Ca into the culture medium at day 5 of culture. In this system, bone resorption is stimulated with PTH and measured by the release of 45Ca into the medium from fetal long bones prelabeled with 45Ca. As the results in Fig. 7show, HP inhibited the PTH-stimulated release of 45Ca in a concentration-dependent manner with an apparent IC_{50} value of 17 ㎍/ml. This value is similar to that obtained for the inhibition of bone resorption with isolated osteoclasts (IC_{50} value of 23 ㎍/ml in our preliminary data). This potency of inhibition of bone resorption was in the range of inhibition of Src activity in cells (17-34 ㎍/ml), suggesting that the observed inhibition of bone resorption may be mediated via Src inhibition. We could also show that HP did not affect

signaling by several growth factors in osteoblastic cells. In addition, the HP did not affect the activity of several serine/threonine protein kinases *in vitro* (data not shown). Thus, HP was not expected to inhibit the serine/threonine-specific cAMP-dependent protein kinase, a major mediator of PTH action in osteoblasts[32]. Furthermore, Src is not required for PTH-mediated adenylate cyclase response in osteoblastic cells[33]. Thus, it is likely that the inhibitory effect of HP in this organ culture system is mediated by Src in osteoclasts.

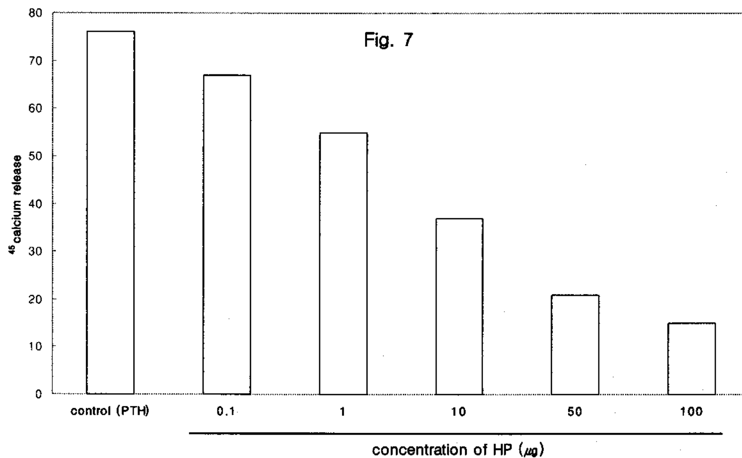

Fig. 7. HP inhibits bone resorption in the fetal long bone organ culture system.
Fetal rat long bones were cultured in the presence of 10 nmol/L human PTH-(1-34) in the presence or absence of HP over the concentration range 0.1-100 ug/ml. Bone resorption was assessed as the percentage release of 45Ca into the culture medium at day 5 of culture. Four experiments are done and the results from a representative experiment are shown as percent release over control for six long bones per group (mean ± SEM, *n* = 6). Open circles show 45Ca release in nontreated controls and PTH-treated samples; closed circles show 45Ca release in samples treated with PTH and HP.

7. HP partially prevents bone loss and rescues bone microarchitectural features in young ovx rats

The ovx rat is often used as a model of postmenopausal osteoporosis[34]. Whether HP partly prevents bone loss in young ovariectomized (ovx) rats, the effect of HP on bone loss in young ovx rats was measured by the trabecular calcium and hydroxyproline content of the distal femur. HP was administered twice a day at doses of 10 and 50 mg/kg orally for 6 weeks, beginning immediately after ovariectomy. We used 8-week-old female rats to assess the effect of orally administered HP on the ovariectomy-induced bone loss. These findings were further corroborated by microtomographic analysis of the architectural changes in vertebral cancellous bone. Trabecular

bone volume, number, separation, and trabecular bone pattern factor all deteriorated in estrogen-depleted, osteopenic rats(Table 1). Treatment with 50 mg/kg HP significantly reduced these effects by approximately 60%. This example shown had a BV/TV fraction very close to the average value of the corresponding group. In ovx animals a stronger prevalence of rod-like structures can be seen than in control or treated animals. This phenomenon was correspondingly reflected by the morphometric parameters in Table 1.

Together, these data show that we identified an Src inhibitor which is potent and specific in enzymatic and cellular assays, and which can inhibit bone resorption *in vitro* and *in vivo*.

Table 1. HP partly prevents ovariectomy-induced changes in the morphometric parameters of vertebral trabeculae in young rats.

Parameter	Control(PBS)	Control (OVX)	OVX +20mg/kg
BV/TV(%)	23.6±2.6	15.3±1.3	21.5±2.1
Tb.N (1/mm)	0.45±0.01	0.28±0.03	0.34±0.02
Tb.Sp (uM)	21.6±2.1	32.6±4.3	28.4±2.5
Tb.Th (uM)	5.5±0.3	4.2±0.4	45.6±5.3**
TBPf (1/mm)	4.3±0.5	9.4±0.5	7.5±0.8*

HP was administered twice a day at doses of 10 and 20 mg/kg orally for 4 weeks, beginning immediately after ovariectomy. The vertebral bone was analyzed with a high-resolution microtomograph and the static bone morphometric parameters were calculated as described. These results are expressed as mean ± SEM. (*, $p<0.05$; **, $p<0.01$ against the OVX control group.)
(OVX, ovariectomized animals; BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness Tb.Sp, trabecular separation; TBPF, trabecular bone factor)

Ⅳ. Discussion

TGF-β strongly suppressed the activities of the cytokines IL-1β, TNF-α and IL-6 actions on stimulation of COX-2 mRNA expression and PGE_2 production in osteoblasts (N=6). It has been reported that TGF-β inhibit prostaglandin production (data not shown).

In this study, we describe a potent inhibitor of the tyrosine kinase Src and demonstrate that it can inhibit bone resorption *in vitro* and *in vivo*. Both in enzymatic and cellular assays HP was selective toward several other tyrosine protein kinases from receptorand nonreceptor families. The different profiles of the Src inhibitors may be useful for addressing various questions in cell biology and in potential treatments for distinct diseases such as cancer, atherosclerosis, immune disorders, and bone metabolism disease.

Our data show that the selective Src inhibitor HP inhibits bone resorption in the fetal long bone system. Obviously, it is hard to totally exclude that inhibition of another protein kinase does not contribute to inhibition of bone resorption. An increase in bone mass results from a positive balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption; thus, an inhibitor of osteoclasts should not inhibit osteoblasts as well.

Administration of IL-1 to mice leads to increased bone resorption, resulting in the release of bone calcium and an increase in serum calcium[35]. The mechanism of IL-1-induced bone resorption probably involves multiple effects on the osteoclast lineage, including stimulation of progenitors, increased differentiation, and activation of mature cells[35]. Treatment of *src-/-*osteopetrotic mice with IL-1 does not induce hypercalcemia, elegantly showing that Src, functional osteoclasts, and bone resorption are all required for IL-1 to increase blood calcium levels[35]. A previous report with the nonspecific Src inhibitor herbimycin A showed a reduction in IL-1-induced hypercalcemia[36].

We have demonstrated IL-6 enhances COX-2 mRNA level as well as synergistically potentiates IL-1β-induced COX-2 mRNA level in mouse gingival fibroblasts which was also reflected by increased PGE_2 production. The data demonstrating reduction of COX-2 mRNA level and subsequently PGE_2 production by tyrosine kinase inhibitors provide also an important role for tyrosine kinases which, by mediating COX-2 at transcriptional and translational level might be a link in the pathogenesis of periodontal diseases. Interleukin-1 together with IL-6 and TNF-αis thought to play a major role in the ovariectomy-induced bone loss[12]. Thus, based on the inhibition of IL-1-induced hypercalcemia with HP, it was expected that the ovariectomy -induced bone loss would be affected as well, but it was possible that theeffect on bone mass could be obscured by unfavorable pharmacokinetics or toxic effects of the compound. We have shown that the loss of the trabecular bone is partly prevented by treatment with the Src inhibitor HERB. Toxicity of HP did not seem to contribute to the positive effects on bone, since the animals survived 6 weeks' treatment with relatively high doses without significant changes in body weight (data not shown). Taken as a whole, our data show that Src inhibitor may be effective in preventing bone loss in animal models, and may be useful for the treatment of conditions associated with bone loss in humans, such as postmenopausal osteoporosis, tumor-induced hypercalcemia, and Paget's disease.

Natural products of plant origin are still a major part of traditional

medicinal systems in developing countries. There is also a resurgence of interest in herbal medicines in western countries as an alternative source of drugs often for intractable diseases such as rheumatoid arthritis[3,4]. A literature survey on the fetal human Hominis Placenta revealed that there is no scientific evidence of its usefulness in the treatment of RA and osteoporosis. The need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

□ 투 고 일 : 2007년 10월 26일
□ 심 사 일 : 2007년 11월 02일
□ 심사완료일 : 2007년 11월 06일

References

1. Shi, X.C. Chinese-English terminology of traditional chinese medicine (1983) Hunan Science Publishing Co. p71-98. China.
2. Kim HM et al. Rehmannia Glutinosa inhibits tumor necrosis factor-alpha and interleukin-1 secretion from mouse astrocytes. Pharmacol Res. 1999:40:171-6.
3. Hee-Tak Hong. Effects of Yukmijihangtang -Jahage extracts on cellular regulation of bone cell and function. Thesis of Ph.D. Dongguk University, Kyungju, Korea. 1998.
4. Phillipson, J.D. Anderson, L. A. Ethnopharmacology and western medicine. J. Ethnopharmacol. 1989: 25, 61-72.
5. T. Yucel-Lindberg et al. Involvement of tyrosine kinases on cyclooxygenase expression and prostaglandin E2 production in human gingival fibroblasts stimulated with interleukin -1βand epidermal growth factor. Biochem. Biophys. Res. Comm. 1999:257:528-532
6. Stein, B., M.S.K. Sutherland. IL-6 as a drug discovery target. Drug Discovery Today 1998:3; 202-213.
7. R. Baron et al. Cellular and molecular biology of the osteoclast. In: M. Noda Editor, Cellular and molecular biology of bone Academic Press, San Diego, CA 1993;445-495.
8. K.A. Hruska et al. Engagement of the osteoclast integrin alpha v beta 3 by osteopontin stimulates phosphatidylinositol 3-hydroxyl kinase activity. Endocrinology 1995:136 :2984-2992.
9. H.M. Kronenberg. Parathyroid hormone: Mechanism of action. In: M.J. Favus Editor, Primer on the metabolic bone diseases and disorders of mineral metabolism, 3rd ed Lippincott-Raven, Philadelphia, PA 1996;68-70.
10. H.L. Uy et al. Use of an *in vivo* model to determine the effects of interleukin-1 on cells at different stages in the osteoclast lineage. J Bone Miner Res 1995:10;295-301.
11. Hla, T., Neilson, K. Human

cyclooxygenase-2 cDNA. PNAS 1992:89;7384-7388.

12. G.A. Rodan, L.G. Raisz, J.P. Bilezikian. Pathophysiology of osteoporosis. In: J.P. Bilezikian, L.G. Raisz and G.A. Rodan Editors, Principles of bone biology Academic Press, San Diego, CA 1996:979-990.
13. G.D. Roodman. Advances in bone biology: The osteoclast. Endocrin 1996:Rev 17;308-332.
14. P. Soriano et al. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 1991:64;693-702.
15. B.F. Boyce et al. Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. J Clin Invest 1992:90;1622-1627.
16. C. Lowe et al. Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. Proc Natl Acad Sci USA. 1992:90;4485-4489.
17. W.C. Horne et al. Osteoclasts express high levels of pp60c-src in association with intracellular membranes. J Cell Biol. 1992:119;1003-1013.
18. S.N. Tanaka et al. Osteoclasts express high levels of pp60c-src, preferentially on ruffled border membrane. FEBS Lett. 1992:313;85-89.
19. S.M. Thomas, J.S. Brugge. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol. 1997:13;513-609.
20. S. Tanaka et al. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature. 1996:383;528-531.
21. I. Nakamura et al. Phosphatidylinositol-3 kinase is involved in ruffled border formation in osteoclasts. J Cell Physiol. 1997:172;230-239.
22. C. Park et al. Inhibitory effects of streptozotocin, tumor necrosis factor-a, and interleukin-1bon glucokinase activity in pancreatic islets and gene expression of GLUT2 and glucokinase. Arch. Biochem. Biophys. 1999:362;217-224.
23 Y. Kuroki et al. Constitutive expression of c-fos gene inhibits type-I collagen synthesis in transfected osteoblasts. Biochem. Biophys. Res. COmm. 1992:182;1389-1394.
24. Sambrook, J et al. Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor, New York. 1988.
25. Z. Songyang et al. Catalytic specificity of protein-tyrosine kinases is critical for selective signaling. Nature. 1995:373;536-539.
26. N.B. Lydon et al. Purification and biochemical characterization of non-myristoylated recombinant pp60c-src kinase. Biochem J. 1992:287;985-993.
27. J.H.M. Feyen et al. N-terminal truncation of salmon calcitonin

leads to calcitonin antagonists. Structure activity relationship of N-terminally truncated salmon calcitonin fragments *in vitro* and *in vivo*. Biochem Biophys Res Commun. 1992:187;8-13.

28. M. Sabatini et al. Infusions of recombinant human interleukin 1a and 1b cause hypercalcemia in normal mice. Proc Natl Acad Sci USA. 1988:85;5235-5239.
29. D. Serban, C. Rordorf-Adam. Quantitation of serum amyloid P component by an enzyme-linked immunoassay. J Immunol Methods. 1986:90;159-164.
30. M. Gunness-Hey, J.M. Hock. Increased trabecular bone mass in rats treated with human synthetic parathyroid hormone. Metab Bone Dis Rel Res. 1984:5;177-181.
31. I.S. Jamall, V.N. Finelli, S.S. Que Hee. A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. Anal Biochem. 1981:112;70-75.
32. J.T. Parsons, S.J. Parsons. Src family protein tyrosine kinases: Cooperating with growth factor and adhesion signaling pathways. Curr Opin Cell Biol. 1997:9;187-192.
33. E. Izbicka et al. pp60 c-src expression and activity in MG-63 osteoblastic cells modulated by PTH but not required for PTH-mediated adenylate cyclase response. J Bone Miner Res. 1994:9;127-132.
34. Blanco, A. et al. Involvement of tyrosine kinases in the induction of cyclo-oxygenase-2 in human endothelial cells.Biochem. J. 1995:312;419-423.
35. G.R. MundyIn. Bone remodeling and its disorders Martin Dunitz Ltd, London, UK 1995:50-53.
36. E. Canalis, S. Rydziel. Platelet-derived growth factor and the skeleton. In: J.P. Bilezikian, L.G. Raisz and G.A. Rodan Editors, Principles of bone biology Academic Press, San Diego, CA. 1996:619-626.