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Suppression of Jahage (*Hominis Placenta)* on prostaglandin E_2 synthesis, alkaline phosphatase and plasminogen activator activities in the mouse calvarial bone cells

Hee-Tak Hong[1], Hee-Jin Kim[1], Dong-Il Kim[1], Moon-Jo Lee[1], Tae-Kyun Lee[1], Dong-Wook Kim[1], Young-Choon Lee[2], Hyung-Min Kim[3] and Cheorl-Ho Kim[1]*

[1]*Department of Biochemistry, Molecular Biology and Gynecology, College of Oriental Medicine, Dongguk University, Kyungju 780-714, Korea;* [2]*Faculty of Life Sciences and Bioresources, Dong-A University, Pusan 604-714, Korea;* [3]*Department of Oriental Pharmacy, Wonkwang University, Iksan 570-749, Korea*

SUMMARY

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 treatment of *Hominis Placenta* (Jahage) extracts (HPE) in the mouse calvarial tissue culture system. HPE was also tested for whether they could inhibit IL-1-induced PGE_2 production. Cell viability was not significantly affected by treatment with the indicated concentration of HPE. The HPE was shown to have the inhibitory effects against the synthesis of PGE_2. We also examined the effect of the pretreatment with HPE. Pretreatment of the Jahage extracts for 1 h, which by itself had little effect on cell survival, did not enhance the synthesis of PGE_2. Furthermore, the Jahage was shown to have the protective effects against plasminogen dependent fibrinolysis induced by the bone resorption agents of IL-1β. Pretreatment of HPE did not enhance the plasminogen dependent fibrinolysis. Pretreatment of HPE for 1 h reduced the bone resorption. These results clearly indicated that HPE plays a key role in inhibition of the osteoclast-mediated bone resorption.

Key words: Jahage (*Hominis Placenta*), bone resorption, osteoporosis, Korean herbal medicine, calvarial bone cells, cell proliferation, osteocalcin, alkaline phosphatase, prostaglandin

INTRODUCTION

Research during the past decade indicates that two interrelated mechanisms are involved in the proteolytic stages of bone resoprtion (Aubauch *et al*., 1981; Vaughan, 1981; Dewhirst *et al*., 1987). One is osteoclast-dependent, calcitonin-sensitive and involves cysteine proteinases as well as matrix metalloproteinase production. The other is mediated by osteoblast, is calcitonin-insensitive and involves the production of collagenase and other gelatinases (type IV collagenases) and stromelysin-1, in response to bone resorptive agents (Meikle *et al*., 1994). 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 (Kuroki *et al*., 1992; Meikle *et al.*, 1992). Osteocalcin and alkaline phosphatase, as phenotype markers of the osteoblast cells, are inducible factors of vitamine D (Evans *et al.*, 1990).

In this study, it was shown than rhIL-1β suppressed the osteocalcin and alkaline phosphatase, as phenotype markers of the osteoblast cells, in association with the stimulation of cell proliferation and the effects of these phenotype markers were strongly antagonized by rhIL-1β in a dose-dependent manner (Hazuda *et al*., 1990). Also, HPE was tested for the inhibitory effects against IL-1β-induced PGE_2 production (Strickler *et al*., 1990; Harrison *et al.*, 1994), plasminogen

*Correspondence: Prof. Cheorl-Ho Kim, Department of Biochemistry, Molecular Biology and Gynecology, College of Oriental Medicine, Dongguk University, Sukjang-Dong 707, Kyungju City, Kyungbuk 780-714, Korea. Tel: 82-54-770-2663; Fax: 82-54-770-2281; E-mail: chkimbio@dongguk.ac.kr

dependent fibrinolysis, and IL-1β-stimulated bone resorption in the mouse calvarial bone cells having both of the osteoblast and osteoclast cells (Heasman *et al.*, 1993). The inhibitory effect of HPE was highly similar to that of calcitonin treatment, indicating these two subjects play some key roles in inhibition of the osteoclast-mediated bone resorption.

On the other hand, to examine the inhibitory effect of some oriental medicinal extracts on the bone resorption and collagenolysis 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 HPE. 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 HPE. From the the results, it was concluded that HPE is highly stable and applicable to clinical uses in osteoporosis.

MATERIALS AND METHODS

Materials and mouse bone cell culture

One hundred grams of the medicinal *Hominis Placenta* was extracted with distilled water for 1 or 2 h at 100°C, respectively, and then centrifuged at 3,0000 rpm for 15 min. The supernatant was stored at 4°C and used as the plant extract. The extracts were pre-warmed to room temperature before use. Alternatively, the aqueous extracts of Jahage (*Hominis Placenta*), which were massproduced and named HPE-7 in the Oriental Herbal Center (OHC) of the Oriental Medical Hospital of Dongguk University College of Oriental Medicine (Kyungju, Korea), were kindly supplied for clinical use.

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, reagents, and Fetal bovine serum (FBS) were from Gibco BRL (Chagrin Falls, OH). Recombinant pure human IL-1β (specific activity 5×10^5 U/mg) was our deposit or was obtained from Genzyme Corp. (Cambridge, MA, USA).

Explants of mouse calvarial bone were cultured as described (Evans *et al.*, 1990). The cells obtained have been routinely characterized and shown to express an osteoblast-like phenotype in culture. Salmon calcitonin was obtained from Armour Pharmaceutical Co. (IL, USA). PGE_2 antibody was purchased from Immunoassay Co. (Tokyo, Japan). [^{3}H]-prostaglandin was purchased from New England Nuclear (Seoul, Korea).

Bone resorption assay

The fetal mouse long bone organ tissue culture system was based on that described by Raisz and Niemann (1969). 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 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°C 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 medium 1, 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-1β ^{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.

Assays of cell proliferation, osteocalcin, alkaline phosphatase and prostaglandin

Cell proliferation was assessed by the incorporation of [^{14}C]-thymidine into materials precipitable by trichloroacetic acid. Cells were pulsed for the final 24 h of a 46 h incubation period. Osteocalcin released into the culture media, over a 72 h incubation period was measured using a specific radioimmunoassay with an antibody raised in rabbits against purified bovine osteocalcin (unpublished data). Results are expressed as ng osteocalcin per μg cell protein. Alkaline phosphatase activity in the solubilized cell layer was measured by monitoring the release of p-nitrophenol from disodium p-nitrophenyl phosphate. The assay buffer consisted of 0.1 M diethanolamine, supplemented with 0.5 M magnesium chloride (pH 10.5). Results are expressed as μmoles per μg cell protein per h. Prostaglandin E_2 (PGE_2) released into the culture medium over a 72 h incubation period was measured by radioimmunoassay using an antiserum with specificity towards PGE_2 (Immunoassay, Co., Tokyo Japan) as descrived in (Aubauch *et al*., 1981). Results are expressed as ng PGE_2 per μg cell protein.

Effects of HPE on IL-1β-induced PGE_2-production, plasminogen activity, osteocalcin production, alkaline phosphatase activity and bone resorption in calvarial bone cells

Two different assays were carried out to assess the activities of Jahage extracts (each 10 μg/ml) on IL-1β-induced PGE_2-production, plasminogen activity, osteocalcin production, alkaline phosphatase activity and bone resorption in the cells, as follows: 1) Experiment-1(post treatment): The mouse calvarial bone cells were treated with IL-1β to induce PGE_2-production, plasminogen activity, osteocalcin production, alkaline phosphatase activity and bone resorption for 24 h, and the treated cells were further treated with Jahage with time courses of 1 and 16 h, and each activity was assayed. 2) Experiment-2 (pretreatment): The mouse calvarial bone cells were initially treated with Jahage for 1 h and further treated with IL-1β to induce for 46 and 56 h. Finally, each activity was assayed (ANOVA).

Statistics

Statistical differences between treatments were determined using analysis of variance.

RESULTS

Effects on PGE_2 production by IL-1β and inhibitory effect of HPE on IL-1β-induced PGE_2 production

IL-1β stimulated the production of PGE_2 in a dose-dependent manner over the concentration range of 0.01 ng-2 ng/ml with a maximal effect being observed at 2 ng/ml (Table 1). The stimulation of cell proliferation was most pronounced at 2.0 ng/ml, while concentrations below 1.0 ng/ml exhibited no detectable activity on the synthesis of PGE_2.

On the other hand, the medicinal extracts of Jahage was tested for whether they could inhibit IL-1β-induced PGE_2 production (Table 1). Cell viability was not significantly affected by treatment with the indicated concentration of the extracts alone (data not shown). The medicinal extracts were shown to have the inhibitory effects against the synthesis of PGE_2. This result indicates that the Jahage extracts could inhibit the cyclooxygenase-2 activity or gene expression of cyclooxygenase-2, which is a mediator of the synthesis of PGE_2 from arachidonic acid. However, their effects were not stringent to protect the synthesis of PGE_2. The PGE_2-induction agents has been known to increase the susceptibility of the calvarial cells against bone resorption, although there are some controversies. Thus, we examined the effect of the pretreatment with a various concentrations of the Jahage extracts then treated the PGE_2-induction agents. Pretreatment of the Jahage extracts for 1 h, which by itself had little effect on cell survival, did not enhance the synthesis of PGE_2. nor significantly reduced the synthesis of PGE_2 by pretreatment (Table 1).

Effect on plasminogen activator activity by rhIL-1β and inhibitory effect of HPE on IL-1β-induced plasminogen activator activity

The plasminogen activator activity of the mouse

Table 1. The effects of rhIL-1β on the prodcution of PGE_2 (A), and inhibitory effect of HPE on IL-1β-induced PGE_2 production (B).

Control	IL-1β (ng / ml)				
	0	0.1	1.0	2.0	10.0
PGE_2 production (pg / g protein)	N.D.	1.59±0.14	24.7±3.1*	87.3±8.4**	162.7±21.5**

Significant difference from control, *P<0.05, **P<0.001.

Exp-1		Exp-2		Exp-1		Exp-2	
1 h	16 h	46 h	56 h	1 h	16 h	46 h	56 h
PGE_2 production (pg / g protein)				PGE_2 production (pg / g protein)			
44.2±5.1*	9.7±1.2**	87.3±10.5*	42.8±3.9**	44.2±5.1*	9.7±1.2**	87.3±10.5*	42.8±3.9**

PGE_2 released into the culture media was measured as described in Materials and Methods. Values represent mean ±S.E.M (n=5). Experiment-1, (Exp-1); Experiment-2 (Exp-2). Significant difference from IL-1β (10 ng / ml)-treated group as a control, *P<0.05, **P<0.001.

Table 2. Stimulation of the plasminogen activator activity by IL-1β and inhibitory effect of HPE on IL-1β-induced plasminogen activator activity

Cont	IL-1β (ng / ml)					Exp-1		Exp-2	
	0	0.01	1.0	2.0	10.0	1 h	16 h	46 h	56 h
Fibrinolysis (%)									
24 h	3.1	2.6±0.1	10.5±0.7*	12.1±1.5*	11.2±1.4*	5.2±0.4#	2.4±0.2##	12.5±1.7	5.3±0.4#

Plasminogen activator activity of mouse osteoblasts induced by rhIL-1β was measured as described in Materials and Methods. The data shown represents the plasminogen dependent fibrinolysis of [^{125}I] -fibrin substrate. Values represent mean ±S.E.M (n=5). Significant difference from control(0), *P<0.001. Significant difference from IL-1β (10 ng / ml)-treated group as a control, #P<0.05, ##P<0.001. Experiment-1, (Exp-1); Experiment-2 (Exp-2)

osteoblast was also stimulated by rhIL-1β in a dose-dependent manner over the dosage range of 0.01 ng -2 ng / ml with a maximal effect being observed at 2 ng / ml (Table 2). The plasminogen activator activity was significantly stimulated compared to that control. Concentrations below 0.1 ng / ml exhibited no detectable activity on the plasminogen activator activity. To examine the anti-plasminogen dependent fibrinolysis of the Jahage extracts on IL-1β-induced plasminogen activator activity in calvarial osteoblast cells, the medicinal extracts were tested for whether they could protect against IL-1β (1 ng / ml)-induced plasminogen dependent fibrinolysis in the mouse calvarial cells (Table 2).

Cell viability was not significantly affected by treatment with the indicated concentration of the extracts alone. Furthermore, the Jahage extracts were shown to have the protective effects against plasminogen dependent fibrinolysis induced by the bone resorption agents of IL-1β. However, their effects were not stringent to protect the plasminogen dependent fibrinolysis. We also examined the effect of the pretreatment with a various concentrations of the Jahage extracts then treated the agents. Pretreatment of the Jahage extracts for 1 h, which by itself had little effect on cell survival, did not enhance the plasminogen dependent fibrinolysis, nor significantly reduced the plasminogen dependent fibrinolysis by pretreatment (Table 2).

Effect of IL-1β on alkaline phosphatase activity and osteocalcin production stimulated by vitamin D

To examine the effects of rhIL-1β on alkaline phosphatase activity stimulated by vitamin D in the mouse osteoblast and osteoclast cells, cells were treated with vitamin D and then the cells were further stimulated by rhIL-1β in a dose-dependent manner. The basal alkaline phosphatase

Table 3. Effect of IL-1β on alkaline phosphatase activity and osteocalcin production stimulated by vitamin D

Control	IL-1β (ng / ml)						
	0	0.001	0.01	0.1	1.0	2.0	10.0
Alkaline phosphatse activity (moles / μg protein / h)	0.021	0.024	0.023	0.018	0.018	0.020	0.021
Osteocalcin (ng / μg cell protein)	0.65	0.64	0.26*	0.38*	0.16**	0.06**	0.02**

Vitamin D (5 μg / ml) was treated to cells and a series of concentration of IL-1β was added to the vitamin D-treated cells. The alkaline phosphatase activity and the osteocalcin production of the solubilized cell layer of the mouse osteoblast cells cells were measured as described in Materials and Methods. Values represent mean ±S.E.M (n=5). Significant difference from vitamin D-treated cells, *P<0.05, **P<0.001

activity of the mouse osteoblast cells was decreased by rhIL-1β over the dose range of 0.01-1.0 ng / ml (Table 3). Concentration below 0.01 ng / ml exhibited no marked inhibitory action on basal enzyme levels. The induction of alkaline phosphatase activity by vitamin D was antagonized by rhIL-1β in a dose depdendent manner over a concentration range of 0.01 ng -2 ng / ml. Lower doses had no obvious effect on the enzyme activity (Table 3). Concentrations below 1.0 ng / ml exhibited no detectable activity on the enzyme activity. On the other hand, when the production of osteocalcin by osteoblast cells were assayed, the productin of osteocalcin stimulated by vitamin D was significantly antagonized by rhIL-1β over the same dose range of 0.01-2.0 ng / ml in a dose depdendent manner (Table 3).

Stimulation of IL-1β on bone resorption and inhibition of IL-1β-stimulated bone resorption by calcitonin and HPE

Treatment of mouse calvarial bone cells with IL-1β resulted in a dose dependent stimulatin of bone resorption. The dose response for stimulating bone resorption differed significantly between the fetal mouse long bone organ tissue culture (unpublished data) and this culture system of mouse calvarial bone cells. As shown in Table 4, human IL-1β is a potent in stimulating bone resorption as measured by means of calcium release when each is normalized to nano gram of amounts. The bone resorption induced by IL-1β appears to be osteoclast-mediaed, since it was largely inhibited by calcitonin treatment, as shown in Table 5. Interestingly, Jahage-extracts were shown to have the inhibiting effects against IL-1β-stimulated bone resorption in the mouse calvarial bone cells having both of the osteoblast

Table 5. Inhibition of IL-1β-mediated bone resorption by HPE

Addition to bone culture	Bone resorbing activity[a] (Calcium release (T%-C%))
None	12.1
Calcitonin (0.5 U / ml)+HPE	6.7*
IL-1β (100 ng / ml)	14.3
Calcitonin+IL-1β+HPE	4.9**
Devitalized bone	2.8

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.
[a]Data shown are means ±S.E.M for quadruplicate determinations.
Bone were devitalized by three cyclyes of freeze-thawing.
*Significantly different from bone treated with IL-1β. *p<0.05. **p<0.01.

Table 4. Dose-dependent bone resorption of IL-1β and inhibitory effect of HPE on IL-1β-induced bone resorption

	IL-1β (ng / ml)						Exp-1		Exp-2	
	0	1	5	10	20	50	1 h	16 h	46 h	56 h
Calcium release (T%-C%)	0.1	1.3	2.2	6.1	6.6	8.7	9.3	3.3*	8.4	3.2*

Bone resorption was measured as percent release of ^{45}Ca during 5 days of mouse calvarial bone cell culture. Each point is the mean paired difference ±S.E. for 5 treatment-control bone pairs. *P<0.05. **P<0.001. Experiment-1, (Exp-1); Experiment-2 (Exp-2)

and osteoclast cells. When we examined the effect of the pretreatment with a various concentrations of the Jahage extracts then treated the agents, pretreatment of the Jahage extracts for 1 h, which by itself had little effect on cell survival, did not enhance the bone resorption, nor significantly reduced the bone resorption by pretreatment. These results are similar to the results from calcitonin treatment (Table 5) and the Jahage extracts play key role in inhibition of the osteoclast-mediated bone resorption induced by IL-1β.

DISCUSSION

In bone resortion reaction, it has been known that the stimulation of prostaglandin E_2 production by IL-1β allows bone breakdown by bone resorption and by stimulating the plasminogen activator activity of osteoblast-cell like cells (Evans *et al.*, 1990). The synthesis of PGE_2 production by IL-1β and resulting stimulation of bone resorption can occur partially via PGE_2-dependent mechanism indicating that the PGE_2 synthesis by osteoblast-like cells in response to IL-1β may contribute to this effect (Kawaguchi *et al*., 1994; Herschman, 1994). Elevated production of IL-1β has been implicated in the pathogenesis od osteoporosis and with humoral hypercalcemia associated with squamous cell carcinomas. The present study therefore provides further support for the role of IL-1β in the bone metabolism process.

When HPE was tested for whether they could inhibit IL-1β-induced PGE_2 production, cell viability was not significantly affected by treatment with the indicated concentration and the HPE showed the inhibitory effects against the synthesis of PGE_2, indicating that HPE could inhibit the cyclooxygenase-2 activity or gene expression of cyclooxygenase-2, which is a mediator of the synthesis of PGE_2 from arachidonic acid (Tai *et al*., 1997). However, their effects were not stringent to protect the synthesis of PGE_2. Pretreatment of HPE, which by itself had little effect on cell survival, did not enhance the synthesis of PGE_2. nor significantly reduced the synthesis of PGE_2 by pretreatment. Next, HPE were tested for whether they could protect against IL-1β-induced plasminogen dependent fibrinolysis in the mouse calvarial cells. Cell viability was not significantly affected by treatment with the indicated concentration of HPE. Also, HPE was shown to have the protective effects against plasminogen dependent fibrinolysis induced by the bone resorption agents of IL-1β. Pretreatment of HPE, which by itself had little effect on cell survival, did not enhance the plasminogen dependent fibrinolysis. Interestingly, HPE showed the inhibiting effects against IL-1β-stimulated bone resorption in the mouse calvarial bone cells having both of the osteoblast and osteoclast cells. When we also examined the effect of the pretreatment with HPE, it reduced the bone resorption. The absolutely same result was also observed in case of calcitonin treatment. Thus, these results suggested that HPE inhibits the bone resorption by inhibiting the osteoclast-mediated bone resorption reaction, which is usually induced by bone resorption agents.

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