Effects of *Bombycis corpus* on Mouse Calvarial Bone Resorption induced by IL-1β

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Abstract

*Bombycis corpus* (BC) is a medicinal silkworm used in Korea for the treatment of various symptoms accompanying hypertension, cerebrovascular disorders and infections. Interleukin-1β (IL-1β) regulates the osteoblast cells derived from mouse calvarial bone explants in vitro. We studied the protective effect of BC extract on bone resorption in mouse calvarial bone cells. When BC extract was tested for whether it could inhibit IL-1β-induced PGE2 production, cell viability was not significantly affected by treatment with the indicated concentration of BC extract. BC extract was shown to have inhibitory effects against the synthesis of PGE2. The effect of pretreatment with BC extract for 1 hr followed by treatment with PGE2-induction agents, had little effect on cell survival, and did not enhance the synthesis of PGE2. Furthermore, BC extract was shown to have protective effects against plasminogen dependent fibrinolysis induced by the bone resorption agents of IL-1β. Pretreatment with BC extract did not enhance the plasminogen dependent fibrinolysis. Finally, calcitonin showed inhibitory activity on IL-1β-stimulated bone resorption in the mouse calvarial bone cells having both osteoblast and osteoclast cells. Seemingly, pretreatment with BC extract for 1 hr reduced the bone resorption. This inhibitory effect of BC extract was highly similar to that of calcitonin treatment. These results clearly indicated that calcitonin and BC extract play key roles in inhibition of the osteoclast-mediated bone resorption.

Key Words: *Bombycis corpus*, bone resorption, plasminogen activator activity, interleukin-1β (IL-1β), prostaglandin E2 (PGE2), osteoblast

Introduction

It has been demonstrated that one cytokine, interleukin-1 (IL-1), can modulate several aspects of the activity of various bone cell types. IL-1 is a potent stimulator of bone resorption *in vitro* [1], an action apparently partially mediated via the stimulation of osteoclast cell formation indicating the modulation of osteoblast cell activity by IL-1β [2]. Previous studies have demonstrated that IL-1β regulates several aspects of the functional activity of human osteoblast-like cells *in vitro* [3,4]. It is also a potent inducer of prostanoids [5,6]. Both prostaglandin-dependent and prostaglandin-independent effects on bone metabolism have been reported. For example, injection of IL-1 above the calvariae of mice caused a short-term prostaglandin-independent stimulation of bone resorption followed by a prolonged increase in resorption, which was prostaglandin-dependent [7]. Osteocalcin and alkaline phosphatase, as phenotype markers of the osteoblast cells [8], are inducible factors of vitamin D. Elevated production of IL-1β has been implicated in the pathogenesis of osteoporosis and with humoral
hypercalcemia associated with some carcinomas.

Several reports have suggested that raw, natural dietary sources may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxidative damage in vitro and in vivo\(^8,9\). Therefore, much attention has been focused on natural antioxidants\(^10,11\).

Batrycated silkworm (*Bombycis corpus*; BC) is an anti-neuropathy drug consisting of the dried larva of silkworm, *Bombycis mori* L., dead and stiffened due to the infection of *Beauveria bassiana* (Bals) Vuill. It is used for treatment of headache, vertigo, tic and skin prurigo, scrofula, tonsillitis, parotitis and purpura\(^12\). In particular it was reported that the extract of BC may exert some anti-aging action and neuroprotective activity\(^13,14\), although little is yet known about the pharmacological effects or active ingredients.

In this study, we have examined whether these effects are exhibited by IL-1\(\beta\) on mouse calvarial osteoblast cells derived from fetal mouse. The medicinal extract of BC was tested for inhibitory effects against IL-1\(\beta\)-induced PGE2 production, plasminogen dependent fibrinolysis, and IL-1\(\beta\)-stimulated bone resorption in mouse calvarial bone cells having both osteoblast and osteoclast cells.

**Materials and Methods**

1. Extract from *Bombycis corpus*

   *Bombycis corpus* (300 g) was purchased from Oriental Medical Hospital, College of Oriental Medicine, Dongguk University and extracted with boiling water for 3 hr. After the extract was centrifuged at 7500 rpm for 30 min, the supernatant was lyophilized. For direct use, the extract solution was stored at 4°C in aliquots.

2. Mouse bone cell culture

   Explants of mouse calvarial bone were cultured as described\(^2\). The cells obtained have been routinely characterized and shown to express an osteoblast-like phenotype in culture. Recombinant pure human IL-1\(\beta\) (specific activity 5 x 10\(^5\) U/mg) was from our deposit\(^15\). Salmon calcitonin was obtained from Armour Pharmaceutical Co. (IL, USA).

3. Materials

   Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from GIBCO-BRL (Grand Island, New York, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), dimethyl sulphoxide (DMSO), bovine serum albumin and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). PGE2 antibody was purchased from Immunoassay Co. (Tokyo, Japan).

4. Cell proliferation

   Cell proliferation was assessed by the incorporation of [\(^3\)H]-thymidine into materials precipitable by trichloroacetic acid. Cells were pulsed for the final 24 hr of a 46 hr incubation period.
5. Determination of cell viability

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the colored formazan product by mitochondrial enzymes in viable cells. The MTT reduction was measured essentially as described previously16).

6. Prostaglandin assay

Prostaglandin E2 (PGE2) released into the culture medium over a 72h incubation period was measured by radioimmunoassay using an antiserum with specificity towards PGE2 (Immunoassay, Co., Tokyo Japan) as described in Ruch’s report8). Results are expressed as ng PGE2 per μg cell protein.

7. Effects of BC-treatment on IL-1β-induced PGE2-production, plasminogen activity and bone resorption in calvarial bone cells

Two different assays were carried out to assess the activities of BC extract (each μg/ml) on IL-1β-induced PGE2-production, plasminogen 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 PGE2-production, plasminogen activity and bone resorption for 24 hr, and the treated cells were further treated with BC with time courses of 1 and 16 hr, and each activity was assayed.

2) Experiment-2 (pretreatment): The mouse calvarial bone cells were initially treated with BC for 1 hr and further treated with IL-1β to induce for 46 and 56 hr. Finally, each activity was assayed.

8. Bone resorption assay

The fetal mouse long bone organ tissue culture system was based on that described by Raisz 17). Fetal bones were labeled with 45Ca by injecting the mother with 200 μCi 45Ca (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 Linpro plates. The shafts were first cultured in 0.5 ml BGJb medium (GIBCO Laboratories, Grand Island, NY) containing 1.0 ml/ml bovine serum albumin, 100 units/ml penicillin G, and 1μg/ml polymyxin B for 1 day to reduce exchangeable 45Ca. One bone from a pair (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 an 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% CO2 incubator at 37°C and 95% humidity with one change of media after 2 days. The percentage of 45Ca released from a bone into the medium during the 5-day culture was determined 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 between treatment and control bone percent 45Ca released during the 5-day culture. Dead bone 45Ca release in this system was approximately 10%. BGJb control 45Ca release was 16-20% and maximum IL-1β 45Ca release was 60-80%. Since “stimulated” release is expressed as the mean difference between paired BGJb 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 45Ca.

9. Protein determination
Protein was determined on each sample with bicinchoninic acid, using bovine serum albumine as the standard.

10. Statistical analysis
Standard procedures were used to calculate means and standard error. Values were compared using Student’s t-test with SigmaPlot for Windows program (Version 3.02, Jandel Corporation). P<0.05 was considered significant.

Results

1. Effect of BC on cell cytotoxicity in cultured osteoblast cells
Cell killing as cytotoxicity was not detected by addition of increasing concentrations of BC extract compared to those of the untreated group. When we measured the amount of MTT reduction from cultured mouse osteoblast cells at 48 hr after peptide treatment, the BC extract did not show MTT reduction compared to the control value at 200 μg/ml concentration (data not shown).

2. Stimulation of cell growth of mouse osteoblasts by IL-1β and inhibitory effect of BC on IL-1β-treated cell proliferation
Proliferation of the mouse osteoblasts was stimulated in a dose-dependent manner by IL-1β over the concentration range of 0.01 ng -5.0 ng/ml (Fig. 1). The stimulation of cell proliferation was maximal at 2.0 ng/ml, while concentrations below 0.1 ng/ml had no detectable effect. For the stimulated osteoblast cells, two different concentrations of BC (20 μg/ml and 40 μg/ml) were applied to IL-1β (2.0 ng/ml)-treated cell proliferation of the cells to examine the regulating effects of BC on unusual cell growth of osteoblasts. It was known that unusual cell proliferation induces cellular differentiation in osteoclastic cell characteristics. Results are shown in Fig. 1, indicating the cell growth was inhibited in two cases of treatment with BC.

3. Inhibitory effect of BC extract on IL-1β-induced PGE2 production in the mouse osteoblasts
IL-1β stimulated the production of PGE2 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 (Fig. 2).

The stimulation of cell proliferation was most pronounced at 2.0 ng/ml, while concentrations below 0.1 ng/ml exhibited no detectable activity on the synthesis of PGE2. To examine the inhibitory effect of BC, the medicinal extract of BC was tested for whether it could inhibit IL-1β-induced PGE2 production (Fig. 2). Cell viability was not significantly affected by treatment with the indicated concentration of the extracts alone (data not shown).

The medicinal extract was shown to have inhibitory effects against the synthesis of PGE2. This result indicates that the BC extract could inhibit the cyclooxygenase-2 activity or gene expression of cyclooxygenase-2, which is a mediator of the synthesis of PGE2 from arachidonic acid. However, the effect was not strong enough to protect the synthesis of PGE2. The PGE2-induction agents have been known to increase the susceptibility of the calvarial cells against bone resorption, although there is some controversy concerning this. Thus, we examined
the effect of pretreatment and posttreatment with concentrations of BC extract (20 μg/ml), then the PGE2-induction agent was added. Pretreatment with BC extracts on IL-1β-induced PGE2 production. PGE2 released into the culture media was measured as described in Materials and Methods. After 1 hr of BC treatment, IL-1β (2.0 ng/ml) was added and PGE2 production was observed after 46 and 56 hr. Values represent mean ± SE (n=3). * p<0.05 compared with 46 hr-treated group; # p<0.05 compared with IL-1β (2 ng/ml)-treated group.

4. Inhibitory effect of BC extract on IL-1β-induced plasminogen activator activity of mouse osteoblasts
The plasminogen activator activity of the mouse osteoblast was also stimulated by IL-1β in a dose-dependent manner over the dosage range of 0.1 ng -2 ng/ml with a maximal effect being observed at 2 ng/ml (Fig. 4). The plasminogen activator activity was significantly stimulated compared to control. Concentrations below 0.01 ng/ml exhibited no detectable activity on the plasminogen activator activity.

To examine the anti-plasminogen dependent fibrinolysis of BC extract (20 μg/ml) on IL-1β-induced plasminogen activator activity in calvarial osteoblast cells, BC extract was tested for whether it could protect against IL-1β (2 ng/ml)-induced plasminogen dependent fibrinolysis in mouse calvarial cells (Fig. 4). Cell viability was not significantly affected by treatment with the indicated concentration of the extract alone.

Furthermore, BC extract was shown to have protective effect against plasminogen dependent fibrinolysis induced by the bone resorption agents of IL-1β. However, this effect was not strong enough to protect the plasminogen dependent fibrinolysis. The effect of pretreatment with a concentration of BC extract (20 μg/ml) before treatment of the agents was examined. Pretreatment with BC extract (20 μg/ml) for 1 hr, which by itself had little effect on cell survival, did not enhance nor significantly reduce the plasminogen dependent fibrinolysis (Fig. 4). To examine the effect of post-treatment with BC, after 56 hr of IL-1β (2.0 ng/ml) treatment, BC (20 μ
g/ml) was further applied, and then plasminogen dependent fibrinolysis was measured after 1 and 16 hr. The effect of posttreatment was also less than that of pretreatment (Fig. 5).

5. Stimulation of IL-1β on bone resorption and inhibition of IL-1β-stimulated bone resorption by calcitonin and BC extract in the mouse calvarial bone cells

Treatment of IL-1β resulted in stimulation of bone resorption. As shown in Fig. 6., human IL-1β is potent in stimulating bone resorption as measured by means of calcium release when each is normalized to nanogram amounts. Interestingly, BC extract was shown to have inhibiting effects against IL-1β-stimulated bone resorption in cells having both osteoblast and osteoclast cells. When we examined the effect of pretreatment with various concentrations of BC extract then treated the agents, pretreatment with BC extract for 1 hr did not enhance nor significantly reduce the bone resorption (Fig. 6), while the effect of post-treatment with BC was lower than that of pretreatment (Fig. 7). The bone resorption induced by IL-1β appears to be osteoclast-mediated, since it was largely inhibited by calcitonin treatment, as shown in Table 1. These results are similar to the results from calcitonin treatment (Table 1) and BC extract plays a key role in inhibition of the osteoclast-mediated bone resorption induced by IL-1β.

Discussion and Conclusion

It is known that IL-1β is reactive to progressive degradation of bone by activating osteoblast cells and by causing progenitor cells to mature. IL-1β stimulated the plasminogen activator activity of the mouse osteoblast cells in a dose-dependent manner. The stimulation of plasminogen activator activity by IL-1β has been observed in several connective tissue cell types, indicating human osteoblast-like cells. IL-1β is a potent stimulator of bone resorption both in vitro and in vivo.
through an action which may be mediated primarily via the osteoblast\(^1\). The observation that IL-1\(\beta\) stimulates the plasminogen activator activity of the mouse osteoblast cells may indicate a potential mechanism for the osteoblast-mediation of bone resorption.

The stimulation of prostaglandin E2 production by IL-1\(\beta\) allows bone breakdown by bone resorption and by stimulating the plasminogen activator activity of osteoblast-cell like cells\(^2\). The synthesis of prostaglandin E2 production by IL-1\(\beta\) and resulting stimulation of bone resorption can occur partially via prostaglandin E2-dependent mechanism. Elevated production of IL-1\(\beta\) has been implicated in the pathogenesis of osteoporosis and with humoral hypercalcemia associated with squamous cell carcinomas. Osteocalcin and alkaline phosphatase are widely accepted phenotype markers of osteoblast cells\(^2\).

When the medicinal extract of BC was tested for whether it could inhibit IL-1\(\beta\)-induced PGE2 production, cell viability was not significantly affected by treatment with the indicated concentration and the medicinal extract was shown to have inhibitory effects against the synthesis of PGE2, indicating that BC extract could inhibit the cyclooxygenase-2 activity or gene expression of cyclooxygenase-2, which is a mediator of the synthesis of PGE2 from arachidonic acid.

Pretreatment with BC extract for 1 hr, which by itself had little effect on cell survival, did not enhance nor significantly reduce the synthesis of PGE2.

BC extract could protect against IL-1\(\beta\)-induced plasminogen dependent fibrinolysis in mouse calvarial cells. Cell viability was not significantly affected by treatment with the indicated concentration of the extract. Also, BC extract was shown to have protective effects against plasminogen dependent fibrinolysis induced by IL-1\(\beta\). Pretreatment with BC extract for 1 hr, which by itself had little effect on cell survival, did not enhance the plasminogen dependent fibrinolysis.

Interestingly, BC extract showed inhibiting effects against IL-1\(\beta\)-stimulated bone resorption. When the effect of the pretreatment with BC extract was assayed, the extract strongly reduced bone resorption. Absolutely the same result was also observed in calcitonin treatment. Thus, these results suggest that BC extract inhibits bone resorption.

<table>
<thead>
<tr>
<th>Addition to bone culture</th>
<th>Bone resorbing activity Calcium release (T% - C%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.8± 1.4</td>
</tr>
<tr>
<td>Calcitonin (0.5 U/ml)</td>
<td>4.7±0.8</td>
</tr>
<tr>
<td>Calcitonin (0.5 U/ml) + BC extract</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>IL-1(\beta)(100 ng/ml)</td>
<td>12.6±0.6</td>
</tr>
<tr>
<td>Calcitonin + IL-1(\beta)</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td>Calcitonin + IL-1(\beta)+ BC extract</td>
<td>3.9±1.2**</td>
</tr>
<tr>
<td>Devitalized bone</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

Bone resorption was measured as percent release of 45Ca during 5 days of culture.
Each point is the mean paired difference ± SE for 5 treatment-control bone pairs.
Data shown are means ± SE for quadruplicate determinations.
Bones were devitalized by three cycles of freeze-thawing.
*., ** Significantly different from bone treated with IL-1\(\beta\). * p<0.05, ** p<0.01.
resorption and osteoporosis by inhibiting the osteoclast-mediated bone resorption reaction, which is usually induced by IL-1β.

References


