The Effect of Kampo Formulae on Bone Resorption in Vitro and in Vivo. II. Detailed Study of Berberine

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We previously isolated berberine from aqueous extracts of tsu-kan-gan, a Kampo formula used for the treatment of osteoporosis. Berberine caused an inhibitory effect on parathyroid hormone (PTH)-stimulated bone resorption in neonatal mouse bone. In this report we describe the inhibitory effect of berberine on the formation of osteoclast-like multinucleated cells (OCLs) in the co-culture of mouse osteoblastic cells and bone marrow cells in the presence of 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], PTH and interleukin-1α (IL-1α). Berberine dose-dependently inhibited the formation of tartrate-resistant acid phosphatase (TRAP)-positive OCLs induced by 1α,25(OH)₂D₃, PTH and IL-1α. We prepared OCLs in the co-culture of osteoblastic cells and bone marrow cells. The effect of berberine on pit formation by OCLs was examined using dentin slices. As OCLs are terminally differentiated multinucleated cells, the survival of OCLs affects the bone-resorbing activity of OCLs. This prompted us to count the number of TRAP-positive OCLs on the slices. Berberine dose-dependently inhibited pit formation and caused a decrease in the number of TRAP-positive OCLs. Calcitonin (CT) inhibited pit formation without affecting the number of OCLs. Berberine accelerated the cell death in OCLs cultivated on a culture plate, but CT did not affect the cell death of OCLs. This suggests that the decrease in the number of OCLs on dentin slices may be due to apoptotic cell death in OCLs. In fact, Hoechst 33258 staining revealed that the treatment of OCLs with berberine resulted in condensed nuclei and a decrease in cell size. Oral administration of berberine (30 and 50 mg/kg/d) to ovariectomized rats prevented a decrease in bone mineral density (BMD) of the lumbar vertebra without affecting the weight of the uterus and plasma concentration of estradiol. These results suggested that berberine prevented a decrease in BMD in vivo by inhibiting osteoclastic bone resorption.

Key words: antosteoporotic; berberine; osteoclast; pit formation; ovariectomy (OVX)

Osteoporosis, which has been defined as a state of low bone mass, is one of the major problems in our aging society. In China and also in Japan, Kampo formulae are used clinically for the treatment of osteoporosis. According to the theory of traditional Chinese medicine, Kampo formulae which tonify the kidney are useful in the treatment of osteoporosis syndrome, including flaccidity-syndrome, bone exhaustion, and the atrophic debility of bones.

In a search for Kampo formulae which have an inhibitory activity on bone resorption, we examined four Kampo formulae which tonify the kidney for inhibitory effects on parathyroid hormone (PTH)-stimulated bone resorption in mouse calvaria organ culture. It was found that the aqueous extract of tsu-kan-gan (TKG) showed the most potent inhibitory effect. When the active constituents of TKG were investigated, berberine was found to exhibit the most inhibitory activity.

It is well known that osteoclastic bone resorption is mediated by two different processes: one is the formation of new osteoclast-like multinucleated cells (OCLs), and the other is the resorbing activity of OCLs.

Thus, we report the effects of berberine on the formation of OCLs and the resorbing activity of OCLs in vitro, as well as the effect of berberine on ovariectomized (OVX) rats in vivo.

MATERIALS AND METHODS

General Newborn ddY mice, 6-week-old male ddY mice and Wistar retired female rats were purchased from Shizuoka Animal Center (Shizuoka, Japan). Fetal bovine serum (FBS) was from JRH Biosciences; alpha modification of eagle's medium (α-MEM) was obtained from ICN Biomedicals, Inc. (Ohio, U.S.A.). PTH (human, 1–34) was purchased from Peptide Institute, Inc. (Osaka, Japan); 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃] was supplied by Kali-Duphar (Weesp, The Netherlands); interleukin-1α (IL-1α) was purchased from Cistron Biotechnology (Pine Brook, NJ, U.S.A.). Collagen gel solutions (Cell matrix, type I-A) were obtained from Nitta Gelatin Co. (Osaka, Japan). Collagenase and bafilomycin A₁ were obtained from Wako Pure Chemical Co. (Osaka, Japan). Calcitonin (CT) was purchased from Asahi Chemical Industry Co. (Osaka, Japan). Dentin slices were kindly provided by Dr. N. Takahashi (Showa University, Japan).

Measurement of Bone Mineral Density (BMD), Serum Estradiol and Uterus Weight Female rats were ovariectomized or given a sham operation (n=8, group 1). OVX rats were assigned to three groups (group 2–4): each group had eight animals. Group 2 was an OVX group, whereas groups 3 and 4 were given berberine.

Berberine suspended in distilled water was administered orally for 4 weeks. At the end of the experimental period, BMD values were measured in the lumbar vertebra using dual energy X-ray absorptiometry (DXA). Blood samples were collected from the heart and centrifuged for 10 min and the serum was separated and analyzed. The uteri of rats were dissected out and weighed.
Formation of OCLs Co-culture with mouse bone marrow cells and osteoblast-like cells was carried out by the method of Takahashi et al.41 Osteoblast-like cells were prepared from 1-d-old mouse calvariae and plated at 10⁴ cells/well in α-MEM containing 10% FBS. Six-week-old male Std-ddy mice were killed by cervical dislocation, and tibiae were aseptically removed. The bone ends were cut off with scissors, and the marrow cavity was flushed with 1 ml of α-MEM using a 25 G needle. Bone marrow cells were washed once with α-MEM, resuspended, and placed at 10⁵ cells/well on cultures of the osteoblast-like cells. Medium was replaced every 2 d. 1α,25(OH)₂D₃ (10⁻⁸ M), PTH (100 ng/ml), IL-1α or various concentrations of compounds were added at the beginning of the culture and at the time of each medium change. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. After culture for 6 d, adherent cells on the well surface were fixed with 10% formalin phosphate-buffered saline (PBS) (pH=7.2) for 10 min and dehydrated with ethanol-acetone (50:50, v/v) for 1 min. The cells were stained for tartrate-resistant acid phosphatase (TRAP) for 12 min at room temperature and cells containing three or more nuclei were counted as OCLs.

Crude OCLs Preparation The assay system for crude OCLs preparation in vitro was slightly modified from the method of Tamura et al.5 In short, primary osteoblast-like cells (2×10⁶ cells/dish) and bone marrow cells (2×10⁶ cells/dish) were co-cultured in α-MEM containing 10% FBS in the presence of 10⁻⁸ M 1α,25(OH)₂D₃. Culture dishes (10 cm diameter) previously coated with 4 ml of collagen gel matrix for 5 min at 37°C were used. After culturing for 8 d, culture media were discarded and the dishes were used for the following experiment.

Pit Formation Assay Dishes were treated with 4 ml of 0.2% collagenase for 20 min at 37°C in a rocker platform. The recovered cell suspensions were gently pipetted for 10—15 times and centrifuged. The supernatant was aspirated and the cells were resuspended in 8.5 ml of 10% FBS medium. Transverse slices of dentin (diameter 4 mm, ca. 200 μm thick) were sterilized in 70% ethanol and left overnight under ultraviolet light. The slices were placed into the wells of a 96-well plate with 0.1 ml 10% FBS medium and prewarmed to 37°C. OCLs (0.1 ml) preparations were transfused onto the slices. After a setting period of 90 min at 37°C, the slices were transferred to 24-well plates containing 0.4 ml 10% FBS, then incubated for 48 h in humidified air at 37°C. Various concentrations of test compounds were added at the beginning of the pit formation assay. At the end of the culture period, slices were placed for 30 min in 1 M NH₄OH and cleaned by ultrasonication to remove adherent cells. The slices were then stained with Mayer hematoxylin solution [hematoxylin, 1 g/l; NaNO₂, 0.2 g/l; AlNH₄(SO₄)₂·12H₂O, 50 g/l; and CH₃COOH, 7.5 ml/l] for 1 min, washed with distilled water and finally air dried. The areas of resorption pits stained with the hematoxylin solution were identified by light microscopy and photographed. The photograph was scanned into an image analysis system. Other slices were stained for TRAP and counted as OCLs, as described above.

Detection of Osteoclast Apoptosis Procedures for the detection of osteoclast apoptosis were slightly modified from the method of Kameda et al.60 Adherent cells were treated with 4 ml of PBS containing 0.001% pronase and 0.02% EDTA at 37°C to remove stromal cells. After 10 min, cells were treated with PBS containing 0.02% collagenase solution for 10 min and then treated with PBS containing 0.2% collagenase solution for 20 min at 37°C on a rocker platform. The cell suspension was centrifuged to obtain osteoclast enriched populations and seeded onto 24-well plates. After an additional 4 h of culture, reagents were added to the plates. After treatment with the reagents for 18 h, cells were fixed with 3.7% formaldehyde solution for 10 min and stained with 1 μM Hoechst 33258 to visualize the location of DNA. The cells were examined by fluorescence microscopy to determine fragmentation of nuclei and/or condensation of chromatin.

Survival of OCLs and Osteoblastic Cells Crude OCLs were placed in 24-well culture plates. After 4 h, reagents were added to the cultures, which were maintained for a further 18 h. At the end of the culture, cells were stained for TRAP, and TRAP-positive OCLs were counted as living osteoclasts. On the other hand, the cell viability of osteoblasts was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) release. Murine primary osteoblastic cells (1.2×10⁵ cells/well) were cultured in 96-well culture plates with compounds in an atmosphere of 5% CO₂ in air. Stock MTT solution (2.5 mg/ml) was added to each well (20 μl/well), and the plates were incubated at 37°C for another 4 h. Acidic isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to each well and mixed thoroughly. The absorbance was read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 620 nm. The medium from the incubated compounds was tested for LDH release by spectrophotometric assay using a commercial kit (Wako Pure Chemical Co., Osaka, Japan).

Statistical Analysis All values were expressed as the means±S.E.M. of 3 or 4 cultures. Significance of the mean differences in each experiment was analyzed by Student’s t-test, and a p value of <0.05 was considered significant.

RESULTS

Effects of Berberine on 1α,25(OH)₂D₃- , PTH- and IL-1α-Induced Osteoclast Formation by Multinucleated Cells (MNCs) Figure 1A shows the effects of berberine on the formation of TRAP-positive OCLs induced by 1α,25(OH)₂D₃. Treatment of cultures with berberine dose-dependently inhibited TRAP-positive OCLs formation at concentrations of 2 and 20 μM. CT, an antosteoporotic reagent, was used as a positive control and it showed a significant inhibitory activity on 1α,25(OH)₂D₃-induced OCLs formation. An increase in the number of TRAP-positive OCLs induced by PTH (100 ng/ml) was also inhibited by adding berberine at concentrations of 2 and 20 μM (Fig. 1B). IL-1α at 2 ng/ml stimulated OCLs formation (56.5±15.2 cells), and its stimulation was depressed completely by berberine (20 μM) as well as indomethacin (0.1 μM) (data not shown).

Reversibility of the Inhibitory Effect of Berberine on 1α,25(OH)₂D₃-Stimulated OCLs Formation In order to examine the reversibility of the inhibitory effect of berberine on OCLs formation, the cells were treated with berberine for the first 4 d, and thereafter berberine was removed from the
Fig. 1. Effects of Berberine on 1α,25(OH)₂D₃ (A) or PTH-Induced (B) Osteoclast-Like Cell Formation
Control: Cultured with 1α,25(OH)₂D₃ (10⁻¹⁰ M) or PTH (100 ng/ml). Samples: cultured with 1α,25(OH)₂D₃ or PTH and each compound. Each value represents the mean ± S.E.M., n=5. Significant decrease in TRAP-positive OCLs compared with 1α,25(OH)₂D₃ or PTH group, *p<0.05, **p<0.01. Experiment two times gave the reproducibility.

Table 1. Recovery of Number of TRAP-Positive OCLs from Inhibition by Berberine at 2 μM

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of TRAP-positive MNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α,25(OH)₂D₃ (0–6 d)</td>
<td>68.30 ± 14.09 (100.0%)</td>
</tr>
<tr>
<td>Berberine (0–6 d)</td>
<td>20.50 ± 4.80 (30.0%)*</td>
</tr>
<tr>
<td>Berberine (0–4 d)</td>
<td>43.33 ± 1.65 (63.3%)</td>
</tr>
</tbody>
</table>

The percentages of TRAP-positive OCLs inhibition by berberine were 30.00 and 63.30% as compared to that of the 1α,25(OH)₂D₃ group, as given in parentheses. Values are the mean ± S.E.M., n=4. Significant difference from 1α,25(OH)₂D₃ alone, *p<0.05.

medium (Table 1). When berberine was cultured with OCLs for 6 d, OCLs formation decreased to about 30% compared with those cultured with 1α,25(OH)₂D₃ alone. When berberine was cultured with OCLs for the first 4 d, OCLs formation decreased to 60% compared to culture with 1α,25(OH)₂D₃ alone. This suggested that the inhibition of OCLs formation by berberine was partially reversible.

Effects of Berberine on Pit Formation and the Number of TRAP-Positive OCLs on Dentin Slices We examined the relationship between pit formation and the number of TRAP-positive OCLs on dentin slices. Graded concentrations of berberine dose-dependently inhibited dentin resorption by OCLs (Fig. 2A). Complete inhibition was obtained at a concentration of 20 μM. Berberine also dose-dependently decreased the number of TRAP-positive OCLs on dentin slices (Fig. 2B). In contrast, CT showed a significant inhibitory effect on dentin resorption, but it had no significant effect on the number of TRAP-positive OCLs (Fig. 2).

Acceleration of Cell Death of OCLs by Berberine, Calcitonin and Baflomycin A₁ We examined the effects of berberine on the cell viability of OCLs. As shown in Fig. 3, surviving OCLs were stained for TRAP, TRAP-positive OCLs
OCLs appeared as red through an inverted microscope, whereas dead OCLs are TRAP-negative and showed reduced cell size and dark intracellular precipitates. TRAP staining revealed that OCLs underwent spontaneous cell death in 10%

Table 2. Effect of Berberine on the Survival of OCLs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surviving TRAP-positive OCLs (%)</th>
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<tbody>
<tr>
<td>After 4 h control</td>
<td>100±2.1</td>
</tr>
<tr>
<td>After 18 h control</td>
<td>81.5±1.6*</td>
</tr>
<tr>
<td>After 18 h 0.2 μM berberine</td>
<td>97.1±2.5</td>
</tr>
<tr>
<td>2 μM berberine</td>
<td>65.6±0.4*</td>
</tr>
<tr>
<td>20 μM berberine</td>
<td>1.62±0.3*</td>
</tr>
<tr>
<td>2 U/ml CT</td>
<td>136±1.9</td>
</tr>
<tr>
<td>100 μM bafilomycin A1</td>
<td>1.04±0.1*</td>
</tr>
</tbody>
</table>

Crude OCLs were cultured in 24-well culture plates with reagents. The number of TRAP-positive viable OCLs was counted and shown as a percentage against the number of control OCLs at the beginning of the culture after 4 h. Data are expressed as the mean±S.E.M., n=4. Superscript are significantly different from the after 4 h control group (×p<0.05) or from the after 18 h control group (+p<0.01).

FBS α-MEM after culture for 18 h, resulting in cell death of about 25% OCLs. Berberine at 2 μM and above significantly stimulated the cell death of OCLs. CT had no significant effect on the cell death, but bafilomycin A1, as well as berberine (20 μM), caused the death of almost all OCLs which were TRAP-negative (Table 2).

Osteoclast Apoptosis Induced by Berberine To determine the mechanism of acceleration of cell death by berberine, we investigated the possibility of berberine-induced osteoclast apoptosis (Fig. 4). We used Hoechst 33258 to visualize the localization of DNA. Bafilomycin A1, a H+ ATPase inhibitor, was reported to trigger apoptotic cell death in osteoclasts. Bafilomycin A1 and berberine caused nuclear condensation and a decrease in cell size, as shown in Fig. 4. Thus, it was suggested that berberine-treated osteoclasts exhibited morphological changes in nuclei characterized by apoptosis.

Effects of Berberine on the Body and Uterine Weight and Lumbar BMD on OVX Rats The body weight of OVX rats increased significantly compared with that of sham-operated rats after 4 weeks. Treatment with berberine did not show any effect on body weight. Uterine weight was reduced in OVX rats as expected, while berberine in the doses used in these experiments showed virtually no effect on uterine weight (Table 3). As shown in Fig. 5, the lumbar BMD were significantly decreased in the OVX group compared with the sham group. Berberine dose-dependently stored this decrease significantly.

DISCUSSION

In the present study, berberine prevented a decrease in BMD in OVX rats and inhibited both the formation of OCLs.

![Fig. 4. Characterization of Apoptosis in Osteoclasts](image)

A: Osteoclast with normal nuclei treated with IL-1α. B: Apoptotic osteoclasts with nuclear fragmentation and decrease in cell size treated with IL-1α plus bafilomycin A1 (100 μM). C: Apoptotic osteoclasts with nuclear fragmentation and decrease in cell size treated with IL-1α plus berberine (20 μM).

Fig. 5. Effect of Berberine on BMD of OVX Mice

Sham, sham operated group; OVX, ovarietomized group. Samples: OVX mice p.o. administrated with berberine at a dose of 30 and 50 mg/kg/d for 4 weeks. Values are expressed as mean±S.E.M., n=8. Significantly different from Sham group, ×p<0.05, significantly different from Sham group, +p<0.01.

Table 3. Effect of Ovariectomy, Berberine on Body Weight, Uterus Weight, Plasma Estradiol and BMD in Rats

<table>
<thead>
<tr>
<th>Measure</th>
<th>Sham-operated</th>
<th>Ovariectomy</th>
<th>Ovariectomy+berberine (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>305±2</td>
<td>301±5</td>
<td>297±4</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>307±3**</td>
<td>329±7</td>
<td>326±6</td>
</tr>
<tr>
<td>Uterus weight (mg/100 g body wt)</td>
<td>180.0±46.6**</td>
<td>59.5±11.0</td>
<td>71.0±20.0</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>37.0±5.5*</td>
<td>8.9±0.5</td>
<td>9.3±40.8</td>
</tr>
</tbody>
</table>

Administration of berberine was started at the time ovariectomy and continued daily for 28 d. Values are means±S.E.M., n=8. Within a row, values with a superscript are significantly different from the ovariectomy (control) group (×p<0.05 and **p<0.01).
and the bone-resorbing activity of OCLs in vitro. Osteoclastic bone resorption is thought to be mediated by two different processes: one is the formation of new OCLs, and the other is the resorbing activity of OCLs.

First, we investigated the effect of berberine on OCLs formation using the co-culture system of mouse osteoblastic cells and bone marrow cells. Takahashi et al. reported that bone-resorbing agents such as 1α,25(OH)2D3, PTH and IL-1α markedly stimulated the formation of OCLs, while CT, a bone resorption-inhibiting hormone, inhibited the OCLs formation stimulated by the three bone-resorbing agents.

Berberine also inhibited OCLs formation stimulated by the three agents. These agents are thought to stimulate OCLs formation by a common mode involving prostaglandin E2 (PGE2), which itself promotes OCLs formation, although the modes of the three agents are different. In our preliminary experiment, berberine at 2 μM showed an inhibitory effect on PGE2 production in osteoblastic cells [control: 2191 ± 111 ng/ml; berberine treatment: 1726 ± 137 ng/ml (2 μM), p < 0.05, 1565 ± 99 ng/ml (20 μM), p < 0.01]. More recently, Fukuoka et al. reported that berberine at 5 μM inhibited the transcriptional activity of cyclooxygenase-2 in human colon cancer cells. The inhibitory effect of berberine on OCLs formation may be due at least partly to the depression of PGE2 production.

It is pertinent to consider that the inhibitory activity of berberine on OCLs formation is sometimes a result of toxic effects. Thus, a recovery experiment was performed but the inhibitory effect of berberine on 1α,25(OH)2D3-stimulated OCLs formation was only in part recovered (Table 1). However, osteoblastic cells are essential for OCLs formation. When we investigated the cytotoxic effect of berberine (2 μM) in osteoblastic cells using MTT and LDH assay, berberine showed no cytotoxicity (data not shown). This suggests that berberine may affect the differentiation of bone marrow cell OCLs rather than affecting the participation of osteoblastic cells in OCLs differentiation.

Secondly, the effect of berberine on the resorbing activity of OCLs was examined by pit formation assay. It has been reported that the decrease in bone resorption associated with the decrease in the number of OCLs can be seen in vivo, and this causes a cessation of bone resorption either during remodeling or after calcium deficiency. As OCLs are the terminally differentiated multinucleated giant cells, and have a short half-life, the survival of OCLs influences bone-resorbing activity. Therefore, we counted the number of TRAP-positive OCLs on dentin slices in addition to the pit formation assay. Berberine was found to inhibit pit formation activity and to decrease the number of TRAP-positive OCLs (Fig. 2). Similar results have been recognized in OCLs treated with iripriflavone, an antiosteoporotic drug, and bafilomycin A1, a vacuolar H+ -ATPase inhibitor. On the other hand, CT inhibited the pit formation activity but showed no decrease in OCLs number (Fig. 2).

Recently, it has been reported that osteoclasts undergo apoptosis during bone remodeling in murine models and in patients with hyperparathyroidism and Paget's disease. Furthermore, it has been reported that OCLs progressively lose viability in vitro via an apoptotic cell death mechanism and CT does not cause OCLs apoptosis.

We investigated the effect of berberine, CT and bafilomycin A1 on the cell viability in cells following the cultivation of OCLs with each compound on a culture plate which did not include dentin slices. As in the previous report, CT did not affect the cell viability, but bafilomycin A1 caused the death of almost all TRAP-positive OCLs. Berberine dose-dependently accelerated the cell death of OCLs. The decrease in the number of OCLs by berberine on dentin slices (Fig. 2) may be due to the acceleration of the cell death of OCLs (Table 2). In addition, bafilomycin A1 was reported to trigger the apoptotic cell death of OCLs. Berberine has also been shown to provoke apoptosis in HL-60 and Bab/c 3T3 cells at about 70—600 μM. From these reports, we investigated using the DNA-binding fluorochrome, Hoechst 33258, to determine whether berberine caused apoptosis in OCLs in the presence of IL-1α which promoted OCLs survival. Berberine-treated OCLs displayed the characterized morphological feature of apoptosis (Fig. 4), as well as an increase in the number of TRAP-negative OCLs. Therefore, it is suggested that berberine causes apoptotic cell death in OCLs and thus inhibits pit formation activity.

When the effect of berberine on OVX rats as a postmenopausal osteoporosis model was investigated, the weight of untreated OVX rats was significantly higher than that of sham rats at the end of the experiment. These results were in accord with those of Omii et al., and this means that OVX operation was successful. Ovariectomy causes atrophy of the uterus and decreased plasma 17β-estradiol, as expected from previous studies. However, the berberine-treated group did not influence these parameters. Nowadays, estrogen supplementation is the most effective therapy for postmenopausal osteoporosis, but the side effect of causing an overgrowth of endometrium, etc., is a serious problem. However, berberine did not show such an estrogen-like side effect (Table 3). Our experiment using a DXA technique showed that berberine prevented the decrease in BMD of OVX rats, indicating that berberine has a preventive activity on bone loss (Fig. 5).

In conclusion, our results indicate that berberine inhibits both the formation of OCLs and bone-resorbing activity in vitro. These inhibitory effects on osteoclastic bone resorption may result in the preventive effect of berberine on the decrease in BMD in vivo.

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REFERENCES