The Effect of Kampo Formulae on Bone Resorption in Vitro and in Vivo

I. Active Constituents of Tsu-kan-gan

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Four water extracts of Kampo formulae (Yi-kkan-sen, Dai-ho-in-gan, Chi-gan, Tsu-kan-gan) were screened for their inhibitory activities on bone resorption induced by parathyroid hormone (PTH) in organ culture using neonatal mouse parietal bones. Among the Kampo formulae, Tsu-kan-gan (TKG) showed the most potent inhibitory activity. We further fractionated the TKG water extract by monitoring the inhibitory activity on bone resorption stimulated by PTH in vitro. The MeOH fraction of the water extract inhibited PTH-stimulated bone resorption, and its inhibitory activity was more potent than those of other fractions. The MeOH fraction was then subjected to Sephadex LH-20 column chromatography to give fractions I, II and III, which were examined for bone resorption activity. Fraction I inhibited PTH-stimulated bone resorption, and its inhibitory activity was more potent than those of the other fractions. Upon oral administration of the three fractions (100 mg/kg/d) to ovariectomized (OVX) mice, fractions I and III prevented the decrease of bone mineral density (BMD) of the lumbar vertebra. Eleven compounds isolated from the MeOH fraction were examined for their inhibitory effect on PTH-stimulated bone resorption. Among them, berberine (1), syringin (3), limonin (4) and mangiferin (10) showed a significant inhibitory effect on bone resorption. In the formation assay of osteoclast-like cells, these compounds decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). The inhibitory effect of TKG on bone resorption may be at least partly due to the inhibitory action of these compounds.

Key words: osteoporosis; Tsu-kan-gan; osteoporosis; Kampo formulae; parathyroid hormone (PTH); ovariectomy (OVX)

Osteoporosis is one of the most important disorders associated with aging and results in bone fractures, with attendant pain, deformity and loss of independence. In a search for natural crude drugs having inhibitory activity on bone resorption, we have reported that several plants widely used in traditional medicine inhibit bone resorption induced by parathyroid hormone (PTH) in organ culture and prevent the decrease in bone mineral density (BMD) of the lumbar vertebra in an ovariectomized animal. Until now, we have reported that the active constituents of Boerhavia repens L., Cimicifuga heracleifolia Komarov, C. foetida L. and Sambucus sieboldiana Blume ex Graebn. show such inhibitory activity on bone resorption.

In China and also in Japan, Kampo formulae, rather than one traditional medicine, are used clinically for the treatment of osteoporosis. Therefore, it is reasonable that extracts of the Kampo formulae be investigated for antosteoporotic activity. According to the theory of traditional Chinese medicine, Kampo formulae which tonify the kidney are useful for treating such osteoporotic syndromes as flaccidity-syndrome, bone exhaustion and atrophic debility of bones.

Thus, we selected four Kampo formulae which tonify the kidney, Yi-kkan-sen (one-kan-sen), Chi-gan (two-kan-sen), Dai-ho-in-gan (three-kan-sen) and Tsu-kan-gan (four-kan-sen). TKG, then studied the inhibitory activity of each extract from the formulae on PTH-stimulated bone resorption. As the extract of TKG was most potent in inhibiting the bone resorption in the four formulae, a detailed study of its active compounds was also conducted.

MATERIALS AND METHODS

General Mice (dd) were purchased from Shizuoka Animal Center (Shizuoka, Japan). PTH (human, 1–34) was purchased from Peptide Institute, Inc. (Osaka, Japan) and Ham’s F-12 medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). CaCl2 was purchased from NEN Research Products (Boston, MA, U.S.A.). All of the natural crude drugs used in the experiments were purchased from Tochimoto Tenkaido (Osaka, Japan). Fetal bovine serum was from JRH Biosciences; fast red-violet LB and naphthol AS-MX were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of Extracts of Four Prescriptions Each Kampo formula was obtained as an extract powder from a mixture of the following medicines according to traditional prescriptions (ratios in parentheses): Tsu-kan-gan (通関丸, TKG): Phellodendri cortex (yellow, 10), Anemarrhenae rhizoma (知母, 10) and Cimicifugae cortex (肉桂, 50); Dai-ho-in-gan (大補陰丸): Phellodendri cortex (yellow, 10), Anemarrhenae rhizoma (知母, 10), Rehmanniae radix (熟地黄, 20) and Testudinis plastrum (龟板, 18); Ni-chi-gan (二至丸): Ecliptae herba (旱蓮草, 10) and Ligustri fructus (女貞子, 10); Yi-kkan-sen (一貫煎): Adenophorae radix (北沙参, 10), Ophiopogonis tuber (麥門冬, 10), Angelicae radix (当帰, 10), Rehmanniae radix (生地黄, 45), Lycii fructus (枸杞子, 19) and Toosendan fructus (川棈子, 4.5). Mixed medicines were extracted with boiling water, and the resulting aqueous extracts were concentrated under reduced pressure and lyophilized.

Constituents of TKG A mixture of Phellodendri cortex
(150 g), Anemarrhenae rhizoma (150 g), and Cinnamomi cortex (7.5 g) was extracted twice with boiling water (600 ml, 1 h, ×2). The total filtrate was concentrated under reduced pressure and lyophilized. The lyophilized material (132.0 g) was extracted successively with MeOH and water to give MeOH (47.0 g), water (28.3 g) and residue fractions (42.8 g), respectively. The MeOH fraction (47.0 g) was chromatographed on Sephadex LH-20 with H₂O, H₂O–MeOH (1 : 1) and MeOH to give fractions I (25.0 g), II (11.4 g) and III (5.1 g).

Fraction I (25.0 g) was rechromatographed on Sephadex LH-20 with H₂O to give fractions I-1 (2.5 g), I-2 (13.0 g) and
Formation of Osteoblast-like Multinucleated Cells (MNCs) Co-culture with mouse bone marrow cells and osteoblast-like cells was carried out by the method of Takanashi et al.17) Osteoblast-like cells were prepared from neonatal mouse calvaria and plated at 10^4 cells/well in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum. Male Std-ddy mice (7-weeks-old) 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 on cultures of the osteoblast-like cells at 10^5 cells/well. Medium was replaced every 2 d. PTH (2.4 \times 10^{-8} \text{m}) and various concentrations of compounds were added at the beginning of the culture and at each time of medium change. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. After 6 d of culture, adherent cells on the well surface were fixed with 10% formalin phosphate-buffered saline (PBS) (pH = 7.2) for 10 min, then permeabilized 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 MNCs.

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

RESULTS

Effects of 4 Kampo Formulae on PTH-stimulated Bone Resorption The water extracts of 4 Kampo formulae, Yi-kkan-sen, Ni-chi-gan, Dai-ho-in-gan and Tsu-kan-gan (TKG), were assayed for PTH-stimulated bone resorption. At a dose of 440 μg/ml, Yi-kkan-sen and TKG showed a significant inhibitory effect, whereas 44 μg/ml only TKG showed a significant inhibitory effect (Fig. 1).

Effects of Various Fractions of Water Extract of TKG on PTH-stimulated Bone Resorption The water extract was partitioned into MeOH, water and residue fractions. At doses of 44 and 440 μg/ml, all fractions showed a significant inhibitory effect. Among them, the MeOH fraction showed the most potent inhibition at a dose of 44 μg/ml (Fig. 2).

Effects of Various Fractions of MeOH Fraction on PTH-stimulated Bone Resorption Sephadex LH-20 column chromatography of the MeOH fraction with water, H₂O–MeOH (1:1) and MeOH gave fractions I, II and III. At a dose of 440 μg/ml, all fractions showed a significant inhibitory effect, whereas at a dose of 44 μg/ml concentration, fraction I showed the most potent inhibitory effect on bone resorption (Fig. 3).

Effects of Various Fractions of MeOH Fraction on BMD in OVX Mice The effects of fractions I, II and III on BMD in OVX mice for 5 weeks were evaluated. OVX caused a significant decrease in BMD (0.058±0.0002 g/cm² in sham-operated mice vs. 0.052±0.0002 g/cm² in OVX mice, p<0.05). A decrease in BMD of OVX mice was significantly prevented by fraction I or II (100 mg/kg/d) (Fig. 4).

Effects of Isolated Compounds on PTH-stimulated Bone Resorption Compounds isolated from TKG were examined for their inhibitory effect on PTH-stimulated bone re-
Fig. 1. Effects of Various Kampo Formulae on PTH-stimulated 45Ca Release from Neonatal Mouse Parietal Bones

Control: Cultured without PTH. PTH: Cultured with PTH (2×10^{-8}M) and each fraction. Significant decrease in 45Ca release compared to PTH group, *p<0.05; **p<0.01. Significant decrease in 45Ca release compared to control group, *p<0.05.

Fig. 2. Effects of Various Fractions on PTH-Stimulated 45Ca Release from Neonatal Mouse Parietal Bones

Control: Cultured without PTH. PTH: Cultured with PTH (2×10^{-8}M) and each fraction. Significant decrease in 45Ca release compared to PTH group, *p<0.05; **p<0.01. Significant decrease in 45Ca release compared to control group, *p<0.01.

Fig. 3. Effects of Fractions I—III of MeOH Fraction on PTH-Stimulated 45Ca Release from Neonatal Mouse Parietal Bones

Control: Cultured without PTH. PTH: Cultured with PTH (2×10^{-8}M). Significant decrease in 45Ca release compared to PTH group, *p<0.05; **p<0.01. Significant decrease in 45Ca release compared to control group, *p<0.01.

Fig. 4. Effect of Fractions I—III of MeOH Fraction on BMD of Ovariectomized Mice

Sham: Sham operated group. OVX: Ovariectomized group. Significant decrease from OVX group, *p<0.05. Significantly different from sham group, *p<0.05.

Table 1. Effect of Isolated Compounds on PTH-Stimulated Bone Resorption

<table>
<thead>
<tr>
<th>Compound</th>
<th>20 μM</th>
<th>200 μM</th>
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<tr>
<td>1</td>
<td>18.7±1.3**</td>
<td>12.3±0.4**</td>
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<tr>
<td>2</td>
<td>51.0±6.5</td>
<td>44.8±4.9</td>
</tr>
<tr>
<td>3</td>
<td>36.3±2.4*</td>
<td>33.5±2.6**</td>
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<td>4</td>
<td>41.5±3.2</td>
<td>32.9±3.7**</td>
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<td>56.1±3.5</td>
<td>65.6±5.0</td>
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<tr>
<td>10</td>
<td>52.0±7.3</td>
<td>46.8±2.3*</td>
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<tr>
<td>Control</td>
<td>25.9±3.4</td>
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Fig. 5. Effects of Various Compounds on TRAP(+) MNCs Formation

Control: Cultured without PTH. PTH: Cultured with PTH (2×10^{-8}M) and each compound. Significant decrease in TRAP(+) MNCs compared to PTH group, **p<0.01. Significant decrease in TRAP(+) MNCs compared to control group, *p<0.05.

strongest inhibitory activity.

Effects of Isolated Compounds on Osteoclast Formation

Figure 5 shows the effects of 1, 3, 4 and 10 on the for-
formation of TRAP(+)MNCs induced by PTH. The number of TRAP(+)MNCs was 3.0 ± 0.5 in the control cultures. Treatment of cultures with 100 ng/ml PTH markedly increased TRAP(+)MNCs (the number: 135.0 ± 4.4). All compounds at 20 μM markedly inhibited the formation of TRAP(+)MNCs.

DISCUSSION

It is well known that PTH plays an important role in the regulation of the calcium metabolism of bone, and it is widely used as a bone resorption stimulator in bone tissue culture systems designed to evaluate the direct effects of compounds under test.18

In this study, we have examined the inhibitory effects of 4 extracts from Kampo formulae on PTH-stimulated 45Ca release from neonatal mouse parietal bones (Fig. 1). Among the extracts, TKG showed the most potent activity on bone resorption. Although Dai-ho-in-gan (大補陰丸) contained Phellodendri cortex (黄柏) and Anemarrhena rhizoma (即母), similarly to TKG, Dai-ho-in-gan was inactive on bone resorption at 440 and 44 μg/ml. From 2 Kampo formulae proportions, TKG contained a total of 97% of Phellodendri cortex and Anemarrhena rhizoma. In contrast, Dai-ho-in-gan contains only 34%; therefore, this difference is one reason TKG showed more highly inhibitory activity than Dai-ho-in-gan. Thus, the water extract of TKG was further fractionated to the MeOH, water and residue fractions, and these fractions were assayed for bone resorption (Fig. 2). Among the three fractions, the MeOH fraction showed the most potent inhibition of PTH-stimulated bone resorption. Thus, the MeOH fraction was subjected to Sephadex LH-20 column chromatography with H2O, H2O-MeOH (1:1) and MeOH to give fractions I, II and III. Among these fractions, fraction I most potently inhibited bone resorption (Fig. 3). It has been reported that an OVX animal can be used as a postmenopausal osteoporosis model.19 Our results showed that fractions I and III prevented the decrease in BMD of OVX mice, indicating that fractions I and III have a preventive activity on bone loss (Fig. 4). As the body weight of fractions I and III-treated mice decreased compared with that of the OVX mice, the preventive action of fractions I and III on bone loss is not thought to be due to an increase in body weight.

In order to investigate the active constituents of the MeOH fraction, eleven major constituents were tested for their inhibitory activity against PTH-induced bone resorption. Among them, berberine (I), syringin (3), limonin (4) and mangiferin (10) showed significant inhibitory effects on bone resorption (Table 1). Only berberine (I), at 20 μM or greater, depressed the 45Ca release of the control. This may mean that berberine (I) inhibits the resorbing activity of preexisting osteoclasts, which are caused by bone-resorbing factors contained in serum and released from bone osteoblasts.

On the other hand, osteoclastic bone resorption is thought to be mediated by two different processes: one is the new formation of osteoclasts, and the other is the resorbing activity of osteoclasts. Our bone resorption assay is thought to include two processes. Newly formed osteoclasts are produced by the fusion of mononuclear osteoclast precursors derived from hematopoietic cells.20 Takahashi et al. developed both a mouse bone marrow cell culture system21 and a co-culture system with mouse bone marrow cells and osteoblast-like cells.20 Osteoclasts formed by these two systems have almost all of the characteristics of authentic osteoclasts.22

In the present study, we investigated the effects of compounds 1, 3, 4 and 10 on the osteoclast-like cell formation using the co-culture system. Four compounds inhibited the formation of TRAP(+)MNCs at the concentration of 20 μM. This result suggests that an inhibitory effect of the four compounds on bone resorption may be, in part, due to their inhibitory effect on osteoclast-like cell formation. However, it is necessary to investigate the effect of these compounds on the bone-resorbing activity of osteoclasts. Nowadays, the pit formation assay of MNCs on dentin slices is useful in studying the resorbing activity of osteoclasts.21

In conclusion, our results suggest that the inhibitory effect of the MeOH fraction of Tsu-kan-gan (TKG) on bone resorption may be, at least partly, due to the inhibitory action of berberine (I), syringin (3), limonin (4) and mangiferin (10).

REFERENCES