Suppressive Effects of the Leaf of Terminalia catappa L. on Osteoclast Differentiation In Vitro and Bone Weight Loss In Vivo

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Summary Oral administration of Terminalia catappa extract (TCE; 1,000 mg/kg) for 5 wk suppressed bone weight loss and trabecular bone loss in ovariectomized mice. An in vitro experiment showed that TCE (1.3–20 µg/mL) did not increase alkaline phosphatase activity, which would indicate osteoclast formation, in osteoblast-like 3T3-L1 cells. On the other hand, TCE (12.5 µg/mL) markedly decreased the number of tartrate resistant acid phosphatase (TRAP)-positive multinucleated cells, which would indicate osteoclast formation, in a co-culture system (bone marrow cells/osteoblastic UAMS-32 cells). A detailed analysis of the stages of osteoclast differentiation revealed that TCE mainly suppressed the differentiation of bone marrow mononuclear cells into osteoclast progenitor cells in the presence of M-CSF and TGF-β. An additional experiment using fractionated TCE revealed that the water-soluble fraction suppressed the bone weight loss in OVX-mice and osteoclast differentiation in vitro. Therefore, the suppressive effects of TCE on bone weight loss in mice might be due to the suppressive effects of highly polar components on the early stage of osteoclast differentiation.

Key Words leaf of Terminalia catappa, prevention of osteoporosis, bone weight loss, osteoclast differentiation, ovariectomized mice

Imbalances in bone remodeling that are caused by an increase in bone resorption relative to bone formation are the cause of most adult skeletal diseases, including osteoporosis and rheumatoid arthritis (1). Estrogen deficiency leads to the most common form of osteoporosis in postmenopausal women. Estrogen replacement therapy is approved for the prevention of bone loss in postmenopausal women as an effective treatment for reducing the incidence of skeletal fractures. However, estrogen usage and compliance are limited due to its numerous undesirable side effects. Therefore, it would be helpful to explore naturally occurring substances, especially those of plant origin that could prevent bone loss without any adverse effects.

In our preliminary study to regulate bone remodeling, Terminalia catappa was shown to have suppressive effects against bone weight loss in ovariectomized mice (unpublished data). The leaves have been used as a folk medicine for treating dermatitis and hepatitis in Asian countries (2). In Taiwan, the fallen leaves of T. catappa have been used to prevent hepatoma and to treat hepatitis, both after drying and shredding and as a drink made by infusion in hot water (3). Some previous studies showed that water extracts of T. catappa leaves could effectively suppress CCL3-induced hepatotoxicity (2, 4) and bleomycin-induced genotoxicity in Chinese hamster ovary cells (5). It has also been reported that the extract of T. catappa leaves shows both antioxidative (5) and anti-inflammatory (6) effects. However, there has been no report of a suppressive effect of T. catappa leaf extract on bone resorption. Therefore, we evaluated the effects of an extract of T. catappa leaves in both in vivo and in vitro experiments.

MATERIALS AND METHODS

Preparation of extract. Terminalia catappa L. leaves were collected in the Kanto area in Japan. Leaves were dried at 70°C for 24 h, and then cut and extracted with boiled water for 1 h. After filtration, the extract was freeze-dried and stored at −20°C. The dried extract was resolved in sterilized and distilled water before being used as a crude extract for in vitro and in vivo experiments.

Evaluation of bone weight loss in ovariectomized mice. Four-week-old female ddY mice (Japan SLC, Inc., Shi-
zuoka, Japan) were divided into groups (8 mice each). Mice were anesthetized with pentobarbital and subjected to ovariectomy or sham operation. Oral administration of the vehicle or TCE dissolved in distilled water was started from the day after surgery and continued at 5 d per week for 5 wk under conditions of 24 ± 1°C and 50 ± 2% humidity with a 12 h light-dark cycle (light on from 8:00 to 20:00). Finally, mice were sacrificed so that their femurs could be extracted (7). The right femurs were used to measure dry weight (dried at 60°C for 24 h) and the dried femurs were ground from the backside using a fine grindstone. The ground femurs were then washed with water, ethanol, and bleaching solution containing 0.5% sodium hypochlorite. The femurs were dried again and observed using a microscope. The amount of cancellous bone was measured on a photograph of the ground bone. All animal studies were performed in compliance with the 2006 guidelines entitled “Notification No. 88 of the Ministry of the Environment in Japan”.

**Cell culture method.** All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in air. Half of the medium was changed every 3 d. Cell viability was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Chemical Co., St. Louis, MO) assay. After culture, cells were treated with 1 mg/mL MTT for 2 h, the precipitated dye was dissolved in dimethylsulfoxide, and the absorbance at 570 nm was measured.

**Alkaline phosphatase (ALP) activity in osteoblast cells** (8). Osteoblast-like MC3T3-E1 cells (Riken Bioresource Center, Ibaraki, Japan) were cultured in α-minimum essential medium (α-MEM, MP Biomedicals, Eschwege, Germany) for the measurement of ALP activity, which was used as an indicator of osteoblast formation (n=3). The cells were seeded at a concentration of 4×10⁴ cells/well on 96-well micro plates with or without TCE (1.3–20 μg/mL). ALP activity was measured using an ALP LabAssay kit 291–58601 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Bone morphogenetic protein 2 (BMP-2) (9), which was used as a positive control in the assay, was purchased from Wako Pure Chemical Industries, Ltd.

**Assay of osteoclast formation in a co-culture system** (10). The effects of *T. catappa* extract (TCE) on osteoclast formation were examined in a co-culture system that included osteoblastic UAMS-32 cells (11) (5×10⁴ cells/mL) and bone marrow cells (1×10⁶ cells/mL) isolated from murine tissues in α-MEM containing 10% FBS in 96-well plates (Corning Inc., Corning, NY). The culture volume was made up to 200 μL per well with α-MEM supplemented with 10% FBS, in the presence of 4.1 ng/mL 1α,25(OH)₂D₃ (Enzo Life Sciences International Inc., Butler Pike, PA) and 352.5 ng/mL PGE₂ (Cayman Chemical, Ann Arbor, MI), with or without TCE. Murine femoral and tibial bone marrow cells were collected from 8-wk-old mice that had been killed by cervical dislocation (12).

**Osteoclast formation in the absence of osteoblast cells** (13). To investigate the effects of TCE on the differentiation of osteoclast cells, bone marrow cells (1×10⁶ cells/mL) were differentiated with two stimulating steps in the absence of osteoblast cells. In the first step, bone marrow cells differentiated to osteoclast progenitor cells after 3 d of incubation in the presence of 50 ng/mL of macrophage colony stimulating factor (M-CSF, R&D Systems Inc., Minneapolis, MN) and 1 ng/mL transforming growth factor-β (TGF-β, BD Bioscience, NJ). In the second step, osteoclast progenitor cells were cultured for 2 d with 50 ng/mL M-CSF and 50 ng/mL of the receptor activator of nuclear factor-κB ligand (RANKL; Wako Pure Chemical Industries, Ltd.) to give pre-osteoclasts. Mature osteoclasts should be formed by the continued culture of pre-osteoclasts. We examined both steps in osteoclast formation with or without TCE.

**Tartrate resistant acid phosphatase (TRAP) staining.** After osteoclast formation, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline (−) for 20 min. After treatment with 95% ethanol for 1 min, the well surface was dried and treated with TRAP staining solution (14), i.e. 0.1 m sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich Chemical Co.), and 1 mg/mL fast red violet LB salt (Sigma-Aldrich Chemical Co.), for 30 min. TRAP-positive multinucleated cells were then counted as osteoclast cells under a microscope.

**Fractionation of TCE.** To identify the active constituents in the crude extract (TCE), an in vivo evaluation of activity was performed following fractionation. TCE (15 g) was partitioned between water and EtOAc (1,000 mL each) three times. Each fraction was then dried and further partitioned to give four fractions, i.e., Hex fraction, 90% aqueous MeOH fraction, BuOH frac-
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Each fraction (equivalent of 1,000 mg/kg TCE) was dried and dissolved in 1% aqueous ethanol for injection orally into OVX-treated mice. Statistical analysis. Data were statistically analyzed using ANOVA followed by a Tukey-Kramer post-hoc analysis to determine the significance of differences in the data among the groups. *p* values less than 0.05 were considered significant. The values are expressed as means±SE.

RESULTS

Effects of TCE in ovariectomized mice

The suppressive effects of TCE on bone resorption due to estrogen deficiency in the experimental animal model were examined. As shown in Fig. 1, TCE significantly prevented both the decrease in bone weight due to OVX and trabecular bone loss at 1,000 mg/kg/d. In the TCE-treated group (500 mg/kg/d), bone morphology was observed with ground femurs. The average dry weight of femurs in the sham-operated group was 49.3±0.7 mg, while that in the ovariectomized group was 42.9±0.7 mg. This decrease in bone weight is thought to be the result of bone resorption enhanced by estrogen deficiency (Fig. 2A). Ovariectomy caused a decrease in the spongiosa of the distal femur. TCE significantly suppressed this decrease at a dose of 500 mg/kg (Fig. 2B and C). On the other hand, TCE did not significantly affect body weight or uterine weight in mice, as shown in Table 1.

Effects of TCE on ALP activity in osteoblast cells

To evaluate the effects of TCE on osteoblastic MC3T3-E1 cells, ALP activity in the medium was measured. Following incubation for 24 h, the control group showed ALP activity of 19.9 units/mL. In the presence of BMP-2 (50 ng/mL), ALP activity was increased to 154.5±6.4% of that in the control. On the other hand, TCE did not produce a significant change in ALP activity (71.3±4.9% of that in the control) even at 20 μg/mL.
Effects of TCE on osteoclast formation

Osteoclast differentiation was estimated by TRAP(+) multinucleated cell formation in UAMS-32 cell/bone marrow cell co-culture. Osteoclast formation was induced by the presence of 1α,25(OH)2D3 and PGE2 in the co-culture. When we did not add 1α,25(OH)2D3 and PGE2, osteoclast formation was not observed. However, when we added 1α,25(OH)2D3 and PGE2, osteoclast formation was noted. With the addition of TCE at 12.5, 25 and 50 mg/mL, osteoclast formation and growth were significantly inhibited (Fig. 3). The number of differentiated osteoclasts induced by 1α,25(OH)2D3 and PGE2 was 296.3±34.7 cells/well. The addition of TCE reduced the number of TRAP(+) multinucleated cells, without cytotoxicity, to 14.1±6.4% at 12.5 mg/mL, 11.3±1.8% at 25 mg/mL and 10.3±4.4% at 50 mg/mL.

Effects of TCE on steps of osteoclast differentiation

The suppressive effects of TCE on the two steps of osteoclast formation were examined. Bone marrow cells differentiated into osteoclast progenitor cells in the presence of TGF-β and M-CSF (step 1). The cells then differentiated into pre-osteoclasts by stimulation with RANKL and M-CSF (step 2). When TCE was added at 6.3, 12.5 and 25 mg/mL to cultured bone marrow cells in the presence of TGF-β and M-CSF (step 1), osteoclast formation was significantly suppressed (Fig. 4A). Furthermore, when TCE was added to cultured cells in the presence of RANKL and M-CSF (step 2), osteoclast formation was significantly suppressed (Fig. 4B).

Cytotoxicity of TCE

In all of the experiments using osteoblast and osteoclast cells, TCE (less than 25 μg/mL) did not affect cell viability in the MTT assay.

Effects of TCE fractions on ovariectomized mice and osteoclast differentiation

Fractionation of TCE gave four fractions: a Hex fraction (0.02 g, 0.15%), 90% MeOH fraction (0.79 g, 5.55%), BuOH fraction (4.81 g, 30.8%) and a water fraction (8.61 g, 60.1%). The suppressive effects of TCE (1,000 mg/kg) on bone weight loss were evaluated in ovariectomized mice. As shown in Fig. 5, the dry weight of femur in the OVX-control group was significantly decreased compared with that in the Sham group for 5 wk. On the other hand, treatment with the water fraction of TCE suppressed this decrease in bone weight. Treatment with the other fractions did not show any significant changes relative to the OVX-control group. The water fraction of TCE had a suppressive effect on osteoclast differentiation to 43.5±2.5% of that in the control at 25 μg/mL without cytotoxicity in an osteoclast formation test in the co-culture system. Since only the water fraction suppressed bone weight loss in OVX-treated mice and osteoclast differentiation in the co-culture system, the water fraction was expected to contain...
levels in osteoblastic MC3T3-E1 cells. Next, we investi-
ated alkaline phosphatase activity compared with control
TCE has osteoblast-activating effects. However, treat-
ment with TCE produced no significant difference in
both in vitro and in vivo (17). We found that
increased in the presence of  TCE, we can assume that
to act as an agonist of  ER or an estrogen promoter in
the uterus weight did not increase under treat-
ment with TCE. TCE is likely to act as an agonist of  ER or an estrogen promoter in
vivo.

The suppressive effects of  TCE on osteoclast formation are expected to be different from those of these other components. TCE reduced osteoclast differentiation in

Bone is continuously shaped and repaired by bone remodeling, which consists of  the processes of  bone resorption by osteoclasts and bone formation by osteo-
blasts (2). Estrogen deficiency promotes bone resorption and trabecular bone becomes thin (15). We found that TCE inhibited bone resorption due to estrogen defi-

cency in this experimental animal model. The suppress-
effects of  TCE were seen at 1,000 mg/kg (equivalent in
to the anti-osteoporotic component.

**DISCUSSION**

The suppressive effects of  TCE on skeletal remodeling are expected to be different from those of these other components. TCE reduced osteoclast differentiation in


dose. As shown in Fig. 2, TCE significantly prevented
decrease in bone weight (Fig. 1), and significant suppression was not observed at a lower
dose. As shown in Fig. 2, TCE significantly prevented
decrease in bone weight (panel A) and trabecular bone loss (panel B) due to OVX. The typical skeletal sta-
tus of  femoral bone is shown in panel C. Based solely on
these results, the cause of  the decrease in bone weight could not be estimated. Mineral components in the
bone and bone density should be measured to clarify
the detailed mechanisms in vivo in further experiments.

Our present in vitro data demonstrated that TCE sup-
pressed bone weight loss in OVX mice.

Based on these suppressive effects of  TCE on bone weight loss in vivo, we anticipated that this extract
either enhances bone formation or suppresses bone resorption. Alkaline phosphatase activity is a widely
used marker of  calcification and osteoblast maturation, both in vitro and in vivo (16). If  this indicator is
increased in the presence of  TCE, we can assume that
TCE has osteoblast-activating effects. However, treat-
ment with TCE produced no significant difference in
alkaline phosphatase activity compared with control
levels in osteoblastic MC3T3-E1 cells. Next, we investi-
gated the effect of  TCE on osteoclast formation in a
UAMS-32 cell/bone marrow cell co-culture. As shown
in Fig. 3, TCE at more than 12.5 μg/mL suppressed osteoclast formation in a co-culture of  bone marrow
cells/osteoblasts. These results from in vitro experi-
ments suggested that the suppressive effects of  TCE on bone weight loss in vivo may have been due to the inhibi-
tion of  bone resorption by osteoclasts rather than by the promotion of  bone formation by osteoblasts. The results of  our experiment could not exclude the possibil-
ity that TCE inhibited bone-marrow stromal cells, which support osteoclast differentiation (17) during incubation. To elucidate the detailed mechanisms in vitro, further investigations in a co-culture system without stromal cells, i.e., UAMS-32 and primary spleen
cells, will be required.

In a co-culture system for osteoclast formation, osteoclast cells produce RANKL in response to a variety
of signals such as 1α,25(OH)2VD3 and parathyroid hor-
mone. RANKL acts as a ligand for the receptor RANK
on osteoclast progenitor cells to induce osteoclast differ-
entiation. Since the amount of  RANKL released in the
co-cultured supernatant did not change in the presence
of  TCE (data not shown), TCE did not appear to suppress
RANKL expression induced by 1α,25(OH)2VD3 and PGE2 in vitro. These results show that TCE may sup-
press osteoclast formation through inhibition of  either of the differentiation steps from bone marrow
cells. Moreover, we investigated the effect of  TCE on osteo-
clast differentiation to determine the detailed mecha-
nism of  its suppressive effects on osteoclastogenesis. In
the presence of  M-CSF and TGF-β, bone marrow cells
differentiate into osteoclast progenitor cells. In the presence
of  M-CSF and RANKL, these progenitor cells differ-
entiate into pre-osteoclasts and mature. As shown in
Fig. 4A and B, TCE markedly inhibited both of  these steps in differentiation. The site of  action of  TCE was
estimated to be the early step in differentiation of  bone marrow cells. For elucidating detailed mechanisms, a further analysis of  differentiation markers such as Mac-
1 or F4/80 may be useful in the differentiation from bone marrow cells to monocytic precursors in the pres-
ence of  IL-3 and M-CSF. Based on these results in vitro, the inhibitory effects of  TCE on osteoclast formation are
not likely to be due to the inhibition of  M-CSF function
or RANKL expression on osteoblast cells.

Estrogen or 1α,25(OH)2VD3 suppresses bone loss to
reduce the number of  osteoclasts in the early stage
before the expression of  RANKL in vivo (18). Estrogen
has been shown to inhibit osteoclast differentiation by
binding to estrogen receptor (ER) in osteoclast or mar-
row cells in vitro (19). The mechanisms of  action of  TCE
are thought to be similar to those of estrogen. However,
since the uterus weight did not increase under treat-
ment with TCE in ovarietomized mice, TCE is likely to
act as an agonist of  ER or an estrogen promoter in
vivo.

The suppressive effects of  TCE on osteoclast formation are expected to be different from those of these other components. TCE reduced osteoclast differentiation in

Fig. 5. Effects of  fractions from TCE on the decrease in
bone weight in OVX mice. Four-week-old female
StddY mice were ovarietomized and treated orally
with sample solutions at 1,000 mg/kg (equivalent in
TCE) 5 d per week for 5 wk. The femoral bone was then
isolated and weighed. Each point represents the
mean±SE (n=6). ***p<0.001 vs ovarietomized con-
trol. Sham: sham-operated control, OVX-Control: ova-
riectomized and treated with vehicle. OVX: ovarieto-
mized and treated with different fractions—A: Hex
fraction, B: 90% MeOH fraction, C: BuOH fraction, and
D: water fraction.
the differentiation stage with M-CSF and TGF-β. Osteoclasts are specialized cells derived from the macrophage hematopoietic lineage, and they undergo differentiation in co-cultures of bone marrow and stromal cells (20, 21). TCE suppressed the early stage of differentiation, in which bone marrow cells are stimulated by M-CSF. Accordingly, TCE should also be able to suppress their differentiation into macrophage. This effect of TCE may be able to influence not only osteoporosis but also various immunologic diseases.

Various dietary components and herbal products have been reported to contain inhibitors of bone resorption. For example, it has been reported that onion, garlic, and parsley can inhibit bone resorption in ovariectomized rats (22, 23). Quercetin from onion extract inhibits the differentiation of osteoclast progenitor into pre-osteoclasts and actin ring formation (24). Essential oils derived from rosemary, thyme and other herbs inhibit osteoclast activity in vitro (25). Ethanol extract from the Formosan orchid Anoectochilus formosanus suppressed bone loss caused by estrogen deficiency through the suppression of RANKL expression that is required for osteoclast formation (26). Hydrolysable tannins that were identified as the main components of TCE have been shown to regulate many biological activities, including antioxidative and antitumor activities (27, 28). Frosin, a hydrolysable ellagitannin, has been shown to inhibit both osteoclast differentiation and function through a mechanism that involves the inhibition of RANKL-induced p38 MAPK and JNK/AP-1 activation as well as actin ring formation (29).

On the other hand, experiments in vivo and in vitro using fractionated TCE revealed that a highly polar component from TCE suppressed bone weight loss in OVX mice and osteoclast formation in a co-culture cell system. The active principle in the water fraction of TCE has been shown to regulate many biological activities, including antioxidative and antitumor activities (27, 28). Frosin, a hydrolysable ellagitannin, has been shown to inhibit both osteoclast differentiation and function through a mechanism that involves the inhibition of RANKL-induced p38 MAPK and JNK/AP-1 activation as well as actin ring formation (29).

In summary, Terminalia catappa extract was shown to suppress the decrease in bone weight caused by estrogen deficiency in mice. The extract was also shown to suppress osteoclast formation at the early stage. The water fraction of TCE showed suppressive activities both in vivo and in vitro. These results revealed that a highly polar component in TCE suppresses the decrease in bone weight in OVX-mice through the inhibition of osteoclast formation.

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