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Regulation of osteoclastogenesis by ganoderic acid DM isolated from *Ganoderma lucidum*

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ABSTRACT

The preventative effects of the ethanol extracts of *Ganoderma lucidum* against the ovariectomized (Ovx)-induced deterioration of bone density in 11-week-old female Sprague Dawley (SD) rats were investigated. The results showed that the *G. lucidum*-treated Ovx rats showed improved bone density compared with the Ovx rats. We studied the effects of *G. lucidum* on osteoclastic differentiation using bone marrow cells and RAW 264 cell D-clone (RAW-D). Differentiation, in response to receptor activator of NF-κB ligand (RANKL) and a tumor necrosis factor α (TNF-α), was inhibited by the ethanol extracts of *G. lucidum* and ganoderic acid DM which was isolated as one of the active compounds by bioassay-guided fractionation. Ganoderic acid DM especially suppresses the expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1). This suppression leads to the inhibition of dendritic cell-specific transmembrane protein (DC-STAMP) expression and reduces osteoclast fusion.

1. Introduction

Osteoporosis is a very common disease accompanied by a high level of bone resorption, especially for postmenopausal women. Estrogen treatment, or hormone replacement therapy, is considered by many physicians to be the best method to prevent bone loss (MacDonald and Gowen, 2001). However, many women do not tolerate the numerous side effects, or are concerned about the possible increased risk of uterine and/or breast cancer (Santell et al., 1997; Scheiber and Rebar, 1999; Anderson and Garner, 1998). There thus remains a need for highly efficacious anti-resorptive agents with excellent safety and tolerability profiles.

Chinese herbal medicines have been widely used for thousands years for the treatment of fractures and joint diseases. *Ganoderma lucidum* is commonly used in traditional Chinese medicine. In the past, the development of herbal anti-osteoporosis formulas was mainly pursued by scientists in Asian countries, including China, Japan and Korea (Hidaka et al., 1999; Xu et al., 2003).

The ovariectomized (Ovx) rat has been the most useful model for the preclinical testing of osteoporosis therapies (Mosekilde, 1995). In this study, we report that *G. lucidum* prevents bone loss in Ovx rats. The osteoclast is the main functional cell which is responsible for bone resorption. Therefore, it is important to study how the ethanol extracts of *G. lucidum* acts on osteoclasts. Furthermore, ganoderic acid DM isolated from the ethanol extracts of *G. lucidum*, blocked osteoclastogenesis.

2. Materials and methods

2.1. Materials

The fruiting body of *G. lucidum* (strain BMC9049) was obtained from Bisoken Inc. (Oita, Japan). The chemicals used were 17β-estradiol (E2), Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), Fetal bovine serum (FBS) (Gibco: activated charcoal, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO) (Wako, Osaka, Japan), glutamine (Nissui, Tokyo, Japan), penicillin, streptomycin, and trypsin (Invitrogen, Carlsbad, CA, USA). Sprague Dawley (SD) rats were obtained from SEAC Yoshitomi (Fukuoka, Japan). 1α,25-dihydroxy vitamin D_3 ($1\alpha,25(OH)_2D_3$) was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, UK), α-minimum essential medium (α-MEM) was from GIBCO BRL (Grand Island, NY, USA), soluble response to receptor activator of NF-κB ligand (sRANKL) was from PeproTech EC Ltd (London, UK), and a tumor necrosis factor α (TNF-α) was obtained from Roche Molecular Biochemical (Mannheim, Germany). Tartrate-resistent acid phosphatase (TRAP) staining kit was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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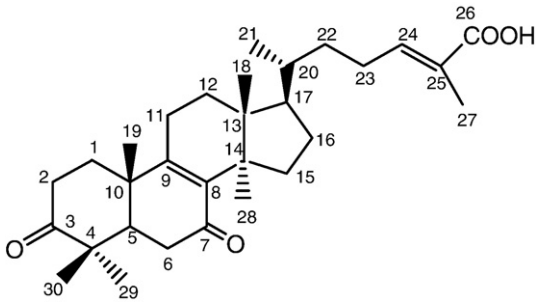

Fig. 1. Chemical structure of ganoderic acid DM.

2.2. Extract preparations of G. lucidum

The dried and chipped fruiting body of *G. lucidum* (15 kg) was extracted with 95% ethanol (126 l) at room temperature for 24 h using a blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and then freeze-dried. The extracts (571.1 g) were stored at −20 °C before the assay.

A portion of the ethanol extracts (50 g) was fractionated into eight fractions (Fr. 1–8) by column chromatography on silica gel (Wakogel C-200, Wako, Osaka, Japan) (2 kg, column size; 20 cm i.d.×150 cm) eluting with an *n*-hexane-ethyl acetate step-gradient. From the further separation of a part of Fr. 5 by a silica gel column guided with a blocking effect of the formation of osteoclast-like multinucleated cells from the RAW 264 cell D-clone, ganoderic acid DM (150 mg, Fig. 1) was isolated as one of the active compounds and identified by comparison with GC-MS data of commercially available standard samples. The extraction rate of ganoderic acid DM is 0.3%. The ^{13}C NMR data for ganoderic acid DM was matched with published data.

2.3. Animals and diets

This experiment was done under the control of the guidelines for Animal experiments in the Faculty of Agriculture and the Graduate Course, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government. Eleven week-old female SD rats (240–260 g) with removed ovaries were purchased from KYUDO Co. Ltd. (Fukuoka, Japan). The animals were kept under standard laboratory conditions, with tap water and regular rat chow available ad libitum, in a 12 h light, 12 h dark cycle, constant temperature at 23.5±1.5 °C. The animals were group housed in plastic cages (one animal per cage). They were divided into 4 weight-matched groups using a randomized complete-block design and given controlled diets for 75 days. Every 2 or 3 days, all animals were observed, and their food intake and body weights were monitored. The treatment groups were divided as follows: ovariectomized (Ovx) control, Ovx+GH (0.3% ethanol extracts of *G. lucidum* in diet), Ovx+GL (0.03% ethanol extracts of *G. lucidum* in diet) and Ovx+E2 (30 μg/kg body weight/day). The composition of experiment diet were; 140 g milk casein, 100 g sucrose, 40 g soybean oil, 35 g AIN-93 mineral mix (Calcium deficiency), 10 g American Institute of Nutrition (AIN)-93 vitamin mix, 1.8 g L-cystine, 2.5 g choline bitartrate, 8 mg *tert*-buthylhydroquinone, 50 g cellulose with or without ethanol extracts of *G. lucidum*, and adjust to 1 kg by adding corn starch. The Ovx+E2 group was subcutaneously injected with E2 solution in 20% polyethylene glycol/0.9% PBS twice per week. It has been reported that Ovx treatment resulted in a significant weight gain caused by an increase in food intake, and that estrogen replacement reduced body weight by decreasing food intake. In addition, the difference in food intake may affect bone metabolism. Taking this issue into consideration, the rats were pair-fed for 75 days. After 75 days, the rats were killed by detruncation. The uterus and other organs were removed, and the wet weights were measured. The right femur was also removed to analyze excised bone mineral density.

2.4. Bone mineral density

Bone mineral density was assessed by ex vivo peripheral Quantitative computed tomography (pQCT) using XCT research SA+. (Stratec Medizintecnik GmbH, Pforzhein Germany). The scans were analyzed using a threshold for delineation of the external boundary and an area peel for subdivision. The trabecular bone density of the right femurs was determined.

2.5. Serum osteocalcin

Peripheral blood for the bone turnover marker determination was collected from the rats. The blood was allowed to clot at room temperature, and the serum was separated by centrifuging at 1015 ×*g* for 10 min at approximately 4 °C. The serum samples were stored frozen (approximately −80 °C) until analysis. Serum osteocalcin was assayed using a rat osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA, USA) according to the manufacturer's instructions.

2.6. Whole bone marrow culture for forming osteoclast-like multinucleated cells

Osteoclast-like multinucleated cells were formed as described previously (Kukita et al., 1993a). Briefly, whole bone marrow cells were flushed out from tibiae and femurs of male SD rats at 4 weeks old, and these cells were cultured for 4 days in 24-multiwell culture plate (1×10^6 cells/well) in α-MEM containing 15% of FBS in the presence of 10% heat-treated ROS17/2.8 cell-conditioned medium (htROSCM) and 10^{-8} M 1α, 25$(OH)_2D_3$. Various concentrations of ethanol extracts of *G. lucidum* or identified chemical components were added to these cultures. After staining for tartrate resistant acid phosphatase (TRAP), TRAP-positive cells having more than 3 nuclei were counted.

2.7. Non adherent bone marrow culture were cultured for forming mononuclear preosteoclasts

Non adherent bone marrow cultures were formed as described previously (Kukita et al., 1993b). Bone marrow cells were flushed out from tibiae and femurs of male SD rats at 4 weeks old. The bone marrow cells were applied to a Sephadex G-10 column previously equilibrated with α-MEM containing 15% of FBS to remove adherent cells, and the non adherent bone marrow cells were collected. These non adherent bone marrow cells did not form any colonies of stromal cells, when they seeded into 24-multiwell culture plate (1×10^6 cells/well) in α-MEM containing 15% FBS in the presence of 10% htROSCM and 10^{-8} M 1α,25$(OH)_2D_3$. Various concentrations of ganoderic acid DM were added to these cultures. After staining for TRAP, TRAP-positive cells having more than 3 nuclei were counted.

Table 1
Weights of body and uterus at the time rats were killed

	Ovx	Ovx+E2	Ovx+GH	Ovx+GL
Initial BW, g	244.2±11.4	243.9±10.4	247.3±7.2	249.0±6.7
Final BW, g	400.3±28.3	354.7±23.8[a]	402.1±19.5	383.9±8.6
Uterus wt., mg	60.6±23.5	180.3±35.0[b]	75.8±9.9	85.1±12.9

Results are given as the mean±SEM. Ovx, ovariectomized; E2, injected with 17β-estradiol; GH, 0.3% ethanol extracts of *G. lucidum* in diet; GL, 0.03% ethanol extracts of *G. lucidum* in diet. Ovx : n=8, E2 : n=9, GH : n=8, GL : n=7, [a]$P<0.05$, [b]$P<0.01$ vs Ovx.

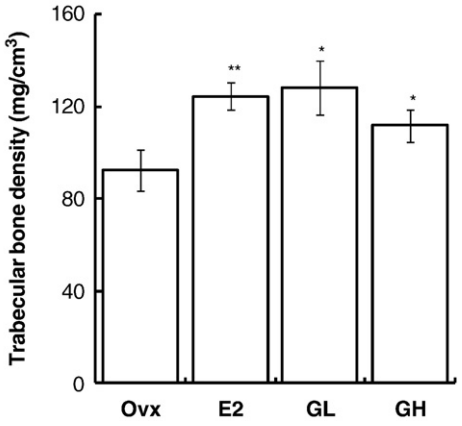

Fig. 2. Influence that ethanol extracts of *G. lucidum* exerts on bone density of Ovx rats. Results are given as the mean±S.E.M. Ovx, ovariectomized; E2, injected with 17β-estradiol; GH, 0.3% ethanol extracts of *G. lucidum* in diet; GL, 0.03% ethanol extracts of *G. lucidum* in diet. Ovx : $n=8$, E2 : $n=9$, GH : $n=8$, GL : $n=7$, $^{*}P<0.05$, $^{**}P<0.01$ vs Ovx.

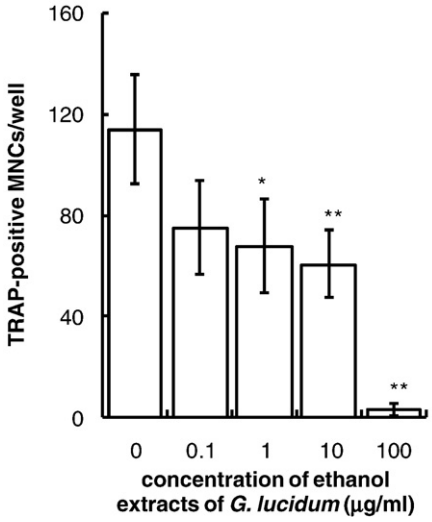

Fig. 4. Influence that ethanol extracts of *G. lucidum* exerts on osteoclastgenesis of whole bone marrow cells. Results are given as the mean±S.D. $n=4$. $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle control.

2.8. Formation of osteoclast-like multinucleated cells from RAW 264 cell D-clone

The osteoclast precursor cell line, RAW 264 cell D-clone, was cultured in α-MEM containing 10% FBS (6.8×10^3 cells in 150 μl/well in 96 well culture plates) for 3 days in the presence of sRANKL (20 ng/ml) and TNF-α (1 ng/ml) as described by Watanabe et al. (2004). In this culture system, numerous osteoclast-like multinucleated cells are formed.

2.9. TUNEL staining

Cultures were fixed in 1% paraformaldehyde/PBS for 10 min at room temperature followed by processing for detecting 3′-hydroxyl terminals of fragmented DNAs according to the manufacturers protocol of ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon Int. Temecula, CA, USA).

2.10. Western blotting

RAW-D cells were stimulated with RANKL and TNF-α in the presence of various concentrations of ganoderic acid DM. After 24 h of culture, cells were solubilized in SDS sample buffer followed by ultrasonication. Protein (20 μg) was loaded on each lane and electrophoresis was performed in 8% SDS–polyacrylamide gel followed by being transferred to the nitrocellulose sheet by use of the semi-dry electrophoretic transfer cell system (Bio-Rad Laboratories, Hercules, CA, USA). After blocking non-specific binding of the antibodies with 3% non-fat dry milk in TBS for 60 min. at room temperature, the nitrocellulose sheet was incubated with anti-NFATc1 antibody or anti-c-Fos antibody or anti-actin antibody (1:100 dilution, Santa Cruz Biotech. Inc. Santa Cruz, CA, USA) at 4 °C for overnight. The membrane filter was rinsed in TBS several times followed by being incubated with hormone replacement therapy-conjugated anti-mouse IgG (1:5000 dilution, Amersham Biosciences, Little Chalfont, UK) at room temperature for 120 min. After being rinsed in TBS-tween 8 times, membrane-bound hormone replacement therapy-conjugated antibodies were detected with ECL system (Amersham Biosciences).

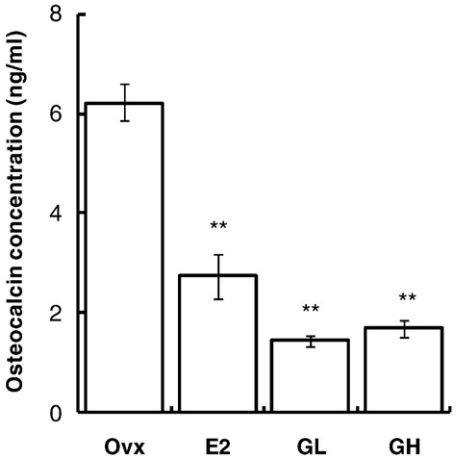

Fig. 3. Influence that ethanol extracts of *G. lucidum* exerts on concentration of osteocalcin in serum of Ovx rats. Results are given as the mean±S.E.M. Ovx, ovariectomized; E2, injected with 17β-estradiol; GH, 0.3% ethanol extracts of *G. lucidum* in diet; GL, 0.03% ethanol extracts of *G. lucidum* in diet. Ovx: $n=8$, E2: $n=9$, GH: $n=8$, GL: $n=7$, $^{*}P<0.05$, $^{**}P<0.01$ vs Ovx.

2.11. Real-time polymerase chain reaction analysis

Total RNA was extracted by use of Isogen (NipponGene, Toyama, Japan) from RAW 264 cell D-clone after being cultured for forming osteoclast-like cells with or without ganoderic acid DM. Complementary DNA was synthesized in a final volume of 20 μl that included 1 μg of total RNA (4–5 μl of 0.2–0.3 μg/μl total RNA), 1 μM oligo-dT 18-mer primer, 10 unit Rnase inhibitor, and 10 units of AMV Reverse Transcriptase (Takara, Japan) according to the manufacturer's instruction. After reverse transcription for 60 min at 45 °C, the samples were heated for 5 min at 95 °C to terminate the reaction. Real-time PCR was performed in a final volume of 10 μl with a Line Gene (Bio flux corporation, Japan). The SYBR Premix Ex Taq kit (Takara, Japan) was used according to the manufacturer's instructions with a final concentration of 0.2 μM each primer. PCR amplification was performed as follows: (i) an initial denaturation at 95 °C for 1 min, (ii) 45 cycles, with 1 cycle consisting of denaturation 95 °C for 15 s, annealing at 60 °C at 15 s, and elongation at 72 °C for 30 s. Amplicon specifity was verified by melting-curve analysis conducted at 65 to 95 °C with stepwise fluorescence acquisition and by 2% agarose gel electrophoresis staining with ethidium bromide. No fluorescence was detected from real-time PCR amplification without a template. Each primer sequence of TRAP (accsession number: M85212) is 5′-CAG CTG TCC TGG CTC AAA A-3′ (forward) and 5′-ACA TAG CCC ACA CCG TTC TC-3′ (reverse), respectively. Each primer sequence of cathepsin K (accession number: AK003425) is 5′-CAG CAG AGG TGT GTA CTA TG-3′

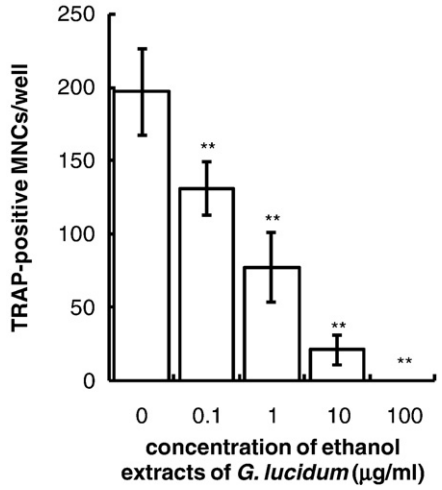

Fig. 5. Influence that ethanol extracts of *G. lucidum* exerts on osteoclastgenesis of RAW-D cells. Results are given as the mean±S.D. $n=4$. *$P<0.05$, **$P<0.01$ vs vehicle control.

(forward) and 5′-GCG TTG TTC TTA TTC CGA GC-3′ (reverse). Each primer sequence of nuclear factor of activated T cells c1(NFATc1) (accession number: NM016791) is 5′-CAA CGC CCT GAC CAC CGA TAG-3′ (forward) and 5′-GGC TGC CTT CCG TCT CAT AGT-3′ (reverse). Each primer sequence of dendritic cell-specific transmembrane protein (DC-STAMP) (accession number: AB109560) is 5′-AAG CGG AAC TTA GAC ACA GG-3′ (forward) and 5′-CCA CAA AGC AAC AGA CTC C-3′ (reverse). Each primer sequence of macrophage fusion receptor (MFR) (accession number: AB018194) is 5′-CTG ATC TGG CTG GAG AAT GG-3′ (forward) and 5′-TCC CAG CAC GGT ATG GTT TC-3′ (reverse). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a reference gene (accession number: BC095932). Each primer sequence is 5′-GCA TTG TGG AAG GGC TCA TGA-3′ (forward) and 5′-GAT GCA GGG ATG ATG TTC TGG-3′ (reverse), respectively. The ratio of gene specific expression was defined as relative expression to the actin expression. The data are three individual run ±S.D.

2.12. Statistical analysis

Data are reported as the mean±S.D. or S.E.M. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test for animal experiments and Student's *t*-test for cell experiments were done to determine any significant difference between the groups. Differences between means at the 5% confidence level ($P<0.05$) were considered to be statistically significant.

3. Results

3.1. The effect of G. lucidum in vivo

All animals gained weight during the course of the experiment, yielding increases of 63.9% (Ovx), 45.4% (Ovx+E2), 62.6% (Ovx+GH), and 54.2% (Ovx+GL) from their initial body weight (Table 1). It should be noted that the sham-operated groups were not examined in this experiment. However, it has been reported that uterine weight was markedly decreased in an Ovx group compared to a sham group, but was increased to the level of the sham group by E2 treatment (Kim et al., 2002). As shown in Table 1, the uterine weight was markedly increased in the E2 treatment compared with the Ovx group. However, the Ovx+GH and Ovx+GL groups showed a slightly higher uterine weight than the Ovx group, respectively, which was far lower than that of the Ovx+E2 group. Also, the weight gain of the Ovx+E2 group was lower than that of the others in the present study. It has been reported that Ovx in rodents leads to weight gain and fat deposition, partially caused by an increase in food intake, and that E2 treatment abolished these effects (Kolta et al., 2003). However in this study, the weight gain in the Ovx rats was mostly caused by the lack of estrogens, because all groups were fed equal amounts of the diet, similar to the previous report (Wu et al., 2004). We investigated the effect of *G. lucidum* on the bone density of ovariectomized rats. By the pQCT analysis of the right femur, significant protection of the trabecular bone density was observed in the Ovx rats treated with E2 and the ethanol extracts of *G. lucidum* at all doses. The trabecular bone density was 92.3±8.8 mg/cm^3 in the Ovx rats. The trabecular bone density was higher to 124.3±5.8 mg/cm^3 (34.6% compared with the Ovx controls) in the E2-treated group, 111.8±7.0 mg/cm^3 (21.0% compared with the Ovx controls) in the Ovx+GH group, and 128.1±11.6 mg/cm^3 (38.8% compared with the Ovx controls) in the Ovx+GL group (Fig. 2). The effect of *G. lucidum* on serum osteocalcin was also evaluated in this study. Compared with the Ovx controls (6.2±0.4 ng/ml), serum osteocalcin was lower to 1.7±0.2 ng/ml (75.9% compared with the Ovx controls) in the Ovx+GL group and 1.4±0.1 ng/ml (79.3% compared with the Ovx controls) in the Ovx+GH group, respectively. In addition, E2 treatment resulted in a 2.7±0.4 ng/ml (61.6% compared with the Ovx controls) reduction in serum osteocalcin (Fig. 3).

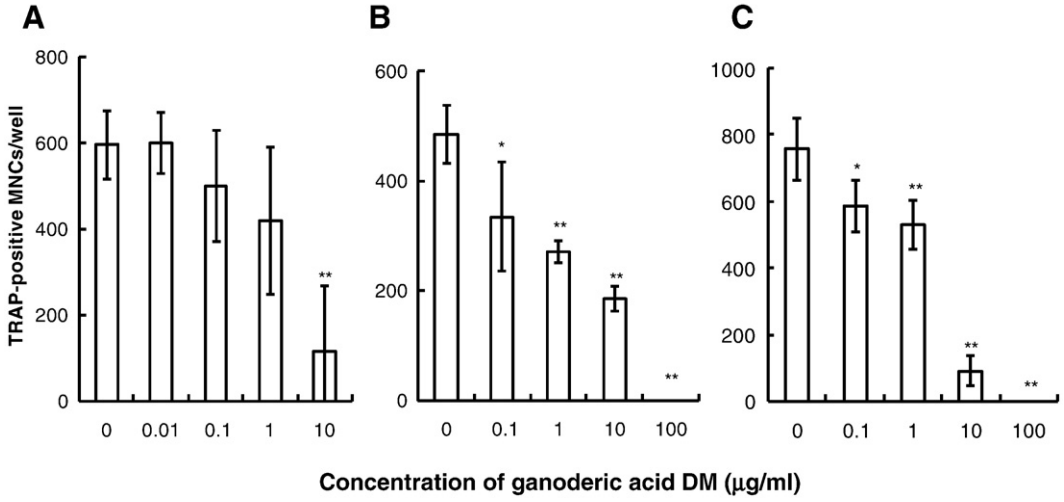

Fig. 6. Influence of ganoderic acid DM on osteoclastgenesis. (A) Influence that ganoderic acid DM exerts on osteoclastgenesis of RAW-D cells. (B) Influence that ganoderic acid DM exerts on osteoclastgenesis of whole bone marrow cells. (C) Influence that ganoderic acid DM exerts on osteoclastgenesis of non adherent bone marrow cells. Results are given as the mean±S.D. $n=4$. *$P<0.05$, **$P<0.01$ vs vehicle control.

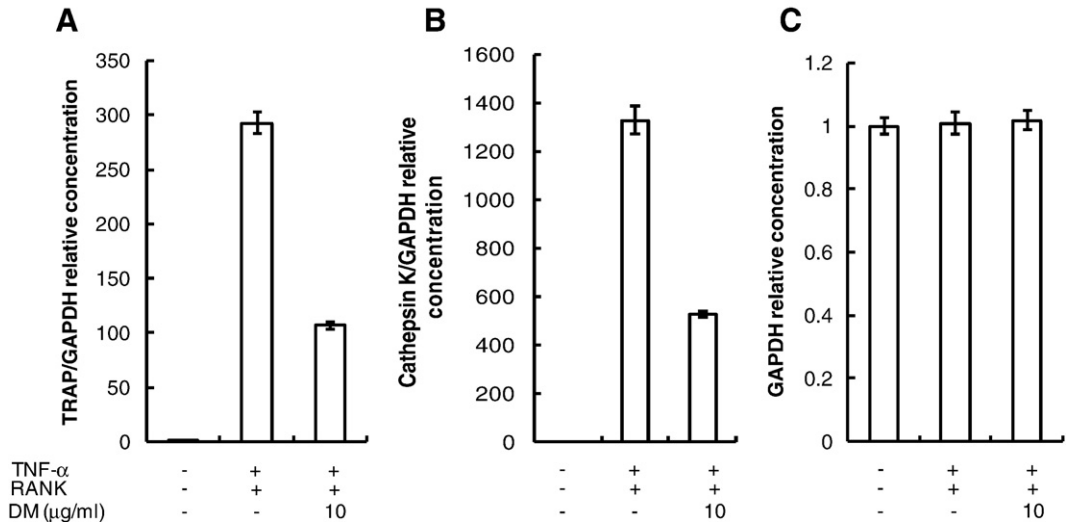

Fig. 7. Influence of ganoderic acid DM on mRNA expression of osteoclast specific genes. (A) The mRNA expression level of TRAP. (B) The mRNA expression level of cathepsin K. (C) The mRNA expression level of GAPDH. DM: ganoderic acid DM.

3.2. Marked inhibition of osteoclastogenesis by ethanol extracts of G. lucidum

We performed a qualitative test in the rat whole bone marrow culture system with respect to the effect of the ethanol extracts of *G. lucidum* on osteoclastogenesis (Fig. 4). An intense inhibitory effect was observed in the ethanol extracts. Approximately 98% inhibition was observed when the cells were treated with 100 μg/ml of the ethanol extracts of *G. lucidum*. To obtain definitive data concerning the direct action of the ethanol extracts of *G. lucidum* on cells in the osteoclast-lineage, we utilized the preosteoclastic cell line RAW 264 cell D-clone (Watanabe et al., 2004). The ethanol extracts of *G. lucidum* clearly suppressed osteoclastogenesis from the RAW 264 cell D-clone (Fig. 5) induced by RANKL and TNF-α. This data show the direct action of the ethanol extracts of *G. lucidum* on osteoclast precursors to suppress osteoclastogenesis.

3.3. Identification of inhibitory compound ganoderic acid DM isolated from G. lucidum ethanol extracts and its effect on osteoclastogenesis

From the separation of the ethanol extracts of *G. lucidum* by a silica gel column guided with a blocking effect of the formation of osteoclast-like multinucleated cells from the RAW 264 cell D-clone, ganoderic acid DM (150 mg, Fig. 1) was isolated as one of the active compounds. Ganoderic acid DM clearly suppressed osteoclastogenesis from the RAW 264 cell D-clone (Fig. 6A). Furthermore, ganoderic acid DM clearly suppressed osteoclastogenesis from the whole bone marrow cells culture and non adherent bone marrow cells culture (Fig. 6B and C). In concordance with the data showing the suppression of osteoclastogenesis by ganoderic acid DM, real-time PCR analysis showed that ganoderic acid DM markedly suppressed the expression of cathepsin K and TRAP mRNA (Fig. 7A and B) without affecting the mRNA level of GAPDH (Fig. 7C) in a system of osteoclastogenesis using the RAW 264 cell-D clone. These results confirmed the ability of ganoderic acid DM as a specific inhibitor of osteoclastogenesis. When we examined the expression of NFATc1, which is believed to be a key transcription factor for osteoclastogenesis, ganoderic acid DM markedly suppressed the expression of NFATc1 and c-Fos in the protein level, as shown in Fig. 8. These data strongly suggest that ganoderic acid DM inhibits osteoclastogenesis through the suppression of transcription factor NFATc1. Real-time PCR analysis showed that ganoderic acid DM markedly suppressed the expression of DC-STAMP mRNA, but not MFR (Fig. 9A and B). However, it should be noted that ganoderic acid DM had no significant effects on the survival of RAW-D cells at the concentration of 10 μg/ml.

4. Discussion

Ovx-rats have been regarded as a model for studying postmenopausal osteoporosis (Kalu, 1991; Frost and Jee, 1992). The ethanol extracts of *G. lucidum* reduced the bone density decrease in Ovx rats. No clear dose–response pattern was observed at any dose in the Ovx+GH and Ovx+GL groups. The reason for this phenomenon remains unclear. It seems that the balance of the composition of the ethanol extracts influence the effect. Genistein stimulated hormone-dependent breast cancer cells (MCF-7 cell) growth at concentrations <10 μmol/L. However, genistein inhibits the MCF-7 cell growth at high concentration (Zava and Duwe, 1997; Miodini et al., 1999; Fioravanti et al., 1998) This situation may be analogous to tamoxifen as noted by Bouker and Hilakivi-Clarke (2000) in that in male rat anterior pituitary cells, low (100 nmol/L) concentrations of tamoxifen increased prolactin secretion but at higher concentrations, estrogen-stimulated prolactin secretion was completely inhibited by tamoxifen (Martinez-Campos et al., 1986). In our study, the low ethanol extracts concentration group showed higher trabecular bone density than the high concentration group. The components of *G. lucidum* may have the same effect as genistein and temoxifen. These components exerted estrogen-like effects at low concentrations which may be considered physiologic concentrations, but at high concentrations exerted other effects, like inhibition of the activity of one or more cellular molecules that control cell signaling, growth and death.

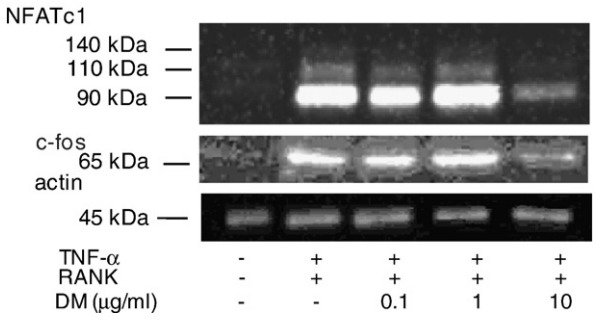

Fig. 8. Influence that ganoderic acid DM exerts on protein expression of NFATc1 and c-Fos. DM: ganoderic acid DM.

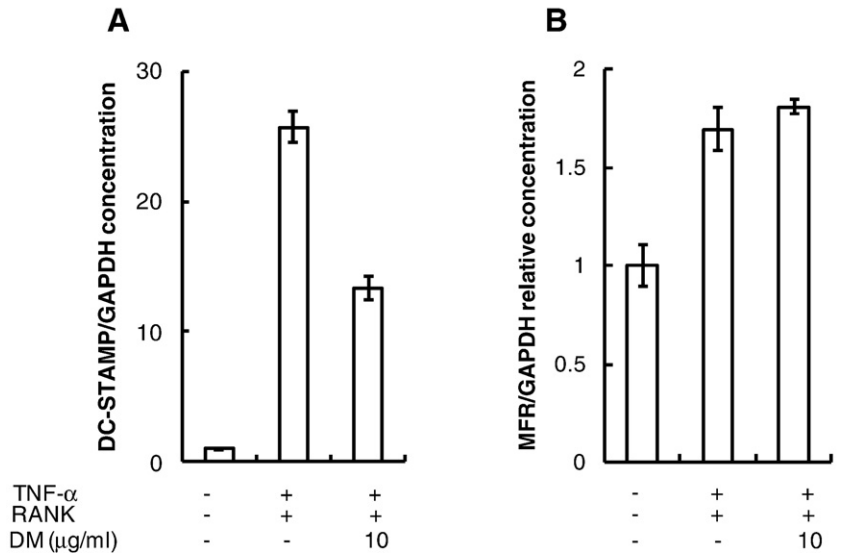

Fig. 9. Influence that ganoderic acid DM exerts on mRNA expression of DC-STAMP and MFR. DM: ganoderic acid DM.

In the present work, we have shown the extremely high potential of the ethanol extracts of *G. lucidum* as a regulator of bone resorption. Real-time PCR analysis revealed that ganoderic acid DM markedly suppressed the expression of cathepsin K without altering the level of GAPDH expression. As cathepsin K is not only a differentiation marker of osteoclasts but also the most important cystein protease for osteoclastic bone resorption (Tezuka et al., 1994), the inhibition of cathepsin K gene expression mediated by ganoderic acid DM could be involved in the marked suppression of osteoclastic bone resorption observed in *G. lucidum*-administrated Ovx rats. Further studies involving a reporter assay are needed to elucidate the underlying mechanism concerning the regulation of cathepsin K gene expression by ganoderic acid DM.

Osteoclast differentiation is induced by macrophage-colony stimulating factor (M-CSF) and RANKL. RANKL binds its cognate receptor RANK and induces expression of c-Fos. The c-Fos induces NFATc1 expression and that c-Fos and NFATc1 cooperatively regulate osteoclastogenesis in response to RANKL stimulation (Fig. 10). Because DC-STAMP is a target of RANKL stimulation, DC-STAMP expression is likely regulated by transcription factors downstream of RANKL-RANK signals, such as c-Fos and NFATc1 (Yagi et al., 2005; Kukita et al., 2004). In our study, ganoderic acid DM significantly suppressed not only c-Fos induction but also NFATc1 upregulation by RANKL. We suggest that the down-regulation of c-Fos by ganoderic acid DM may be the major factor of suppression of NFATc1 expression by RANKL (Fig. 10). This inhibition resulted in a strong inhibition of DC-STAMP expression during osteolastogenesis without influencing the expression of MFR. MFR is also reported as an important receptor for the cell fusion of osteoclasts and macrophages (Vignery, 2005, 2000). Ganoderic acid DM has the possibility of reducing cell fusion by especially suppressing the expression of DC-STAMP. Ganoderic acid DM isolated from *G. lucidum* negatively regulated osteoclast differentiation by RANKL in RAW-D, whole bone marrow cells and non adherent bone marrow cells.

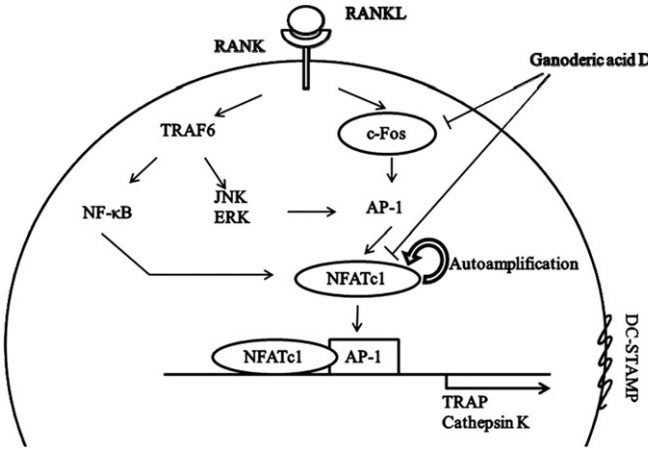

Fig. 10. The essential signaling pathway for normal osteoclastogenesis.

In summary, our results suggest that the ethanol extracts of *G. lucidum* have a bone-protecting effect, without exhibiting a substantial effect on the uterus, in Ovx rats. The active compound, ganoderic acid DM, especially suppresses the expression of c-Fos and NFATc1. This suppression regulates the DC-STAMP expression and reduces osteoclast fusion. Although additional experiments are needed to confirm its efficacy in disease conditions *in vivo*, we propose a possibility that *G. lucidum* can be used in development of a therapeutic drug for osteoporosis.

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