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ARTICLES

Genistein and Zinc Synergistically Stimulate Apoptotic Cell Death and Suppress RANKL Signaling-Related Gene Expression in Osteoclastic Cells

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Abstract The effects of the combination of genistein and zinc, which have an anabolic effect on bone metabolism, on osteoclastic cells in mouse marrow culture system in vitro was investigated. The macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages were cultured in the presence of M-CSF (10 ng/ml) and receptor activator of nuclear factor kappaB (NF-kB) ligand (RANKL; 50 ng/ml) for 4 days. The osteoclastic cells formed were further cultured in medium containing either vehicle, genistein, zinc sulfate (zinc), or genistein plus zinc with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h. The number of osteoclastic cells was significantly decreased with culture of genistein (10^{-6} M) plus zinc (10^{-5} M) in presence or absence of M-CSF and RANKL for 24 or 72 h as compared with the value for genistein or zinc alone. Agarose gel electrophoresis showed the presence of low-molecular weight deoxyribonucleic acid (DNA) fragments of adherent cells cultured with genistein (10^{-6} M) plus zinc (10^{-5} M) for 24 or 72 h, indicating that the combination of two chemicals induces apoptotic cell death. Such an effect was not seen in the case of each chemical. Genistein plus zinc-induced decrease in osteoclastic cells were significantly inhibited in the presence of caspase-3 inhibitor (10^{-8} or 10^{-7} M). Culture with genistein (10^{-6} M) plus zinc (10^{-5} M) for 72 h caused a significant increase in caspase-3 mRNA expression in the presence or absence of M-CSF and RANKL as compared with the value for each chemical alone. Genistein plus zinc-induced increase in caspase-3 mRNA expression was completely inhibited in the presence of cycloheximide (10^{-7} M), an inhibitor of protein synthesis, or 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 10^{-6} M), an inhibitor of transcription activity. The mRNA expression of tartrate-resistant acid phosphatase (TRACP) or cathepsin K was significantly decreased with culture of genistein plus zinc in the presence of M-CSF and RANKL for 72 h as compared with genistein or zinc alone. Nuclear factor of activated T cells c1 (NFATc1) mRNA expression was significantly decreased with culture of genistein plus zinc in the presence of M-CSF and RANKL for 24 or 72 h as compared with each chemical alone, while NF-kB mRNA expression was not significantly changed. This study demonstrates that the combination of genistein and zinc has potent stimulatory effects on apoptotic cell death and suppressive effects on osteoclastic cell function. J. Cell. Biochem. 101: 529–542, 2007. © 2007 Wiley-Liss, Inc.

Key words: genistein; zinc; osteoclast; apoptosis; caspase-3; catepsin K; NFATc 1

Aging induces a decrease in bone mass. Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem that are expected to increase dramatically as the population ages. Bone loss with increasing age may be due to decrease bone formation and increase bone resorption. Pharmacological and nutritional factors may prevent bone loss with increasing age [Bonjour et al., 1996; Yamaguchi et al., 2000].

Genistein is a natural isoflavonoid phytoestrogen found in Leguminosae. The isoflavonoid has been shown to have a strong inhibitory effect on protein tyrosine kinase [Liu et al., 1994; Spinozzi et al., 1994], and it can produce cell cycle arrest and apoptosis in leukemic cell [Bergamaschi et al., 1993; Spinozzi et al., 1994]. Genistein has also been demonstrated to have an anabolic effect on bone metabolism, suggesting its role in the prevention of osteoporosis [Arjmandi et al., 1996; Blair et al., 1996;

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Yamaguchi and Gao, 1997, 1998a]. Genistein has a stimulatory effect on bone formation and mineralization in tissue culture in vitro [Yamaguchi and Gao, 1997, 1998a; Gao and Yamaguchi, 1999a], and it can stimulate protein synthesis in osteoblastic cells [Yamaguchi and Sugimoto, 2000; Sugimoto and Yamaguchi, 2000a; Sugimoto and Yamaguchi, 2000b]. Moreover, genistein has been shown to inhibit osteoclastic bone resorption by preventing the formation of osteoclast-like cells from bone marrow cells [Yamaguchi and Gao, 1998b; Gao and Yamaguchi, 1999b], and the apoptosis of mature osteoclasts is induced by genistein through the Ca^{2+} signaling mechanism [Gao and Yamaguchi, 1999c; Yamaguchi, 2000]. Genistein may be of significance in the prevention of bone loss with increasing age.

Zinc is known to be an essential trace element for the growth of humans and other animals [Prasad et al., 1961; Burt et al., 1975]. Zinc deficiency results in a retardation of bone growth [Hsieh and Navia, 1980; Oner et al., 1984], suggesting that the element is required for the growth, development, and maintenance of healthy bone. The pathophysiologic role of zinc in osteopenia and osteoporosis has also been shown. Bone zinc content is reduced with increasing age [Yamaguchi and Ozaki, 1990] and skeletal unloading in rats [Yamaguchi and Ehara, 1995]. Osteoporosis patients have been shown to have the lower levels of skeletal zinc than normal individuals [Reginster et al., 1998]. Woman with osteoporosis excretes a great amount of zinc in urine [Herzberg et al., 1990]. Zinc supplementation has been shown to have preventive and therapeutic effects on bone loss [Higashi et al., 1993; Kishi et al., 1994]. Zinc may have a role as a nutritional and pharmacologic tools in the prevention of osteoporosis with increasing age [Yamaguchi, 1998; Yamaguchi and Uchiyama, 2003]. Zinc has been shown to stimulate osteoblastic bone formation [Yamaguchi et al., 1987; Yamaguchi and Hashizume, 1994; Yamaguchi and Fukagawa, 2005] and inhibit osteoclastic bone resorption [Yamaguchi et al., 1992; Kishi and Yamaguchi, 1994; Holloway et al., 1996].

The anabolic effect of genistein on bone metabolism has been shown to enhance synergistically with zinc treatment in vitro and in vivo [Gao and Yamaguchi, 1998; Yamaguchi and Gao, 1998a; Yamaguchi et al., 2000]. The prolonged intake of dietary genistein and zinc affects on bone loss induced by ovariectomy [Ma et al., 2000] and circulating bone biochemical markers in aged individuals [Yamaguchi et al., 2005]. The combination of genistein and zinc may have a potent-preventive effect on osteoporosis. The cellular mechanism by which the combination of genistein and zinc has a synergistic-anabolic effect on bone metabolism has not been fully clarified.

More recent study shows that genestein and zinc synergistically enhance gene expression and mineralization in osteoblastic MC3T3-E1 cells [Uchiyama and Yamaguchi, 2006]. This study, furthermore, was undertaken to determine whether the combination of genistein and zinc has a potent-suppressive effect on osteoclastic cells.

MATERIALS AND METHODS

Chemicals

α-Minimal essential medium (α-MEM) and penicillin-streptomycin (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), genistein, synthetic human parathyroid hormone (1–34) (PTH), cycloheximide, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), macrophage colony-stimulating factor (M-CSF, mouse), and receptor activator of NF-kB ligand (RANKL, mouse) were obtained from Sigma (St. Louis, MO). Caspase-3/CPP 32 inhibitor W-1 (caspase-3 inhibitor), zinc sulfate, and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used were glass distilled.

Animals

Male mice (ddY strain; 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus, and 0.012% zinc, and given distilled water. Mice were killed by exsanguinations.

Marrow Culture

Bone marrow cells were isolated from mice. Bone ends of the femur were cut off, and the marrow caving was flushed with 1 ml of α-MEM. The marrow cells were washed with α-MEM and cultured in the same medium containing 10%

heat-inactivated FBS at 1.0×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37°C. The cells were cultured for 3 days; then 0.2 ml of the old medium was replaced with fresh medium, and the cultures were maintained for an additional 4 days. Various concentrations of genistein and/or zinc were added to the culture medium containing either vehicle or PTH (10^{-7} M), with an effective concentration at the beginning of the cultures and at the time of medium change.

Preparation of Osteoclasts

Bone marrow cells were isolated from mice for studies on primary osteoclast precursor, as reported elsewhere [Ogasawara et al., 2004]. Briefly, bone ends of the femur were cut off, and the marrow cavity was flushed with 1 ml of α-MEM. The marrow cells were washed with α-MEM and cultured in the same medium containing 10% FBS and M-CSF (10 ng/ml of medium) at 1.5×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturates atmosphere containing 5% CO_2 and 95% air at 37°C. After 2 days, adherent cells were used as the M-CSF-dependent bone marrow macrophage (M-BMM) after washing out the non-adherent cells included lymphocytes. The cells were then cultured for 24 h in medium containing 0.5% FBS, and after culture the medium was exchanged. The cells were furthermore cultured in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml) for 4 days to generate mature osteoclasts.

To determine the effect of genistein or zinc on the cell death and apoptosis of osteoclasts, the osteoclasts were cultured in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genestein (10^{-6} M) plus zinc (10^{-5} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h.

Enzyme Histochemistry

After culture, cells adherent to 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [Burstone, 1958; Minkin, 1982]. Briefly, cells were washed with Hanks' balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone [1958]. The fixed cells were incubated for 12 min at room temperature (25°C) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate, and red violet LB salt (Sigma) as stain for the reaction product in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Analysis of DNA Fragmentation

The osteoclastic cells formed were cultured for 24 or 72 h in medium containing either vehicle, genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml). The culture supernatant was removed, and adherent cells were than lysed in 10 mM Tris-HCl, pH 2.4, 10 mM EDTA (neutralized), and 0.5% Trion X-100. Low-molecular weight DNA fragments were separated by electrophoresis in 1.5% agarose gel [Preaux et al., 2002]. Gels were visualized by ethidium bromide staining with an UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan). DNA content in the cell lysate was determined by the method of Ceriotti [1995].

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. After the M-BMM were cultured in α-MEM containing 0.5% FBS for 24 h and were further cultured in the presence of M-CSF (10 ng/ml) and RANKL (25 ng/ml), the generated osteoclasts were cultured for 24 or 72 h in medium containing either vehicle, genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M). After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isoprepanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR) was preformed with a Titam™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of mouse

caspase-3 cDNA were: 5′-GCTCTGGTACG-GATGTGGACGCA-3′ (sense strand, positions 254–276 of cDNA sequence) and 5′-CT-CAATGCCACAGTCCAGCTCCG-3′ (antisense strand, positions 560–582) [Juan et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 329-bp sequence from the mRNA of mouse caspase-3. Primers for amplication of mouse TRACP cDNA were; 5′-CAACGGCTACTTGCGGTTTC-3′ (sense strand, positions 963–982) and 5′-TGTGGGAT-CAGTTGGTGTGG-3′ (antisense strand, positions 1,281–1,300) [Cassady et al., 1993]. The pair of oligonucleotide primers was designed to amplify a 388-bp sequence from the mRNA of mouse TRACP. Primer for amplication of mouse cathepsin K cDNA were; 5′-CAGCAGGATG-TGGGTGTTCA-3′ (sense strand, positions 47–66) and 5′-ACACTGGCCCTGGTTCTTGA-3′ (antisense strand, positions 442–461) [Rantakokko et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 415-bp sequence from the mRNA of mouse cathepsin K. Primers for amplication of mouse NFATc1 cDNA were; 5′-GGCGAAGCCCAAGTCTCTTT-3′ (sense strand, positions 1,484–1,503) and 5′-ACCACCAGCCACGAGATCAT-3′ (antisense strand, positions 1805–1824) [Li et al., 1995]. The pair of oligonucleotide primers was designed to amplify a 341-bp sequence from the mRNA of mouse NFATc1. Primers for amplication of mouse NF-kB cDNA were; 5′-TCCACGAGGCAGCACATAGA-3′ (sense strand, positions 3,028–3,047) and 5′-CCAAA-GGTGCTGGAGAATCG-3′ (antisense strand, positions 3,291–3,310) [Ghosh et al., 1990]. The pair of oligonucleotide primers was designed to amplify a 283-bp sequence from the mRNA of mouse NF-kB cDNA. Glyceroald-hyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. Primers for amplication of G3PDH cDNA were; 5′-GATTTGGCCGTATCGGAC-GC-3′ (sense strand) and 5′-CTCCTTGGAGGC-CATGTAGG-3′ (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977-bp sequence from the mRNA of rat G3PDH.

RT-PCR was performed using reaction mixture (20 μl) containing 2 μg of total RNAs, supplied RT-PCR buffer, Titam^{TM} enzyme mix (AMV and Expand^{TM} High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 35 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 60 s at 68°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Image density was quantified with a Fluoro Imager SI (Amersham Pharmacia Biotech.).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *P*-value less than 0.05 was considered to indicate a statistically significant difference. Also, we used an ANOVA multiple comparison test to compare the treatment groups.

RESULTS

Effects of Genistein and Zinc on Osteoclastogenesis

The effects of genistein or zinc on the bone-resorbing factor-induced osteoclast-like MNC formation in the mouse marrow culture system were examined. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, genistein (10^{-7}–10^{-5} M), zinc (10^{-7}–10^{-4} M), or genistein (10^{-7}–10^{-5} M) plus zinc (10^{-7}–10^{-4} M) in the presence or absence of PTH (10^{-7} M). The number of TRACP-positive MNCs was significantly increased in the presence of PTH (Fig. 1). When whole bone marrow cells were cultured on a dentin slice, the PTH (10^{-7} M)-induced TRACP-positive MNCs stimulated the number of resorption pits formed over 7 days, as shown in previous studies [Kishi and Yamaguchi, 1994]. TRACP-positive MNCs were not formed appreciably in the control culture without PTH. The presence of genistein (10^{-7}–10^{-5} M) or zinc (10^{-5} or 10^{-4} M) in the culture medium caused a significant decrease in the number of TRACP-positive MNCs stimulated by PTH. The combination of genistein (10^{-6} M) plus zinc (10^{-5} M) or genistein (10^{-5} M) plus zinc (10^{-4} M) caused a significant decrease in TRACP-positive MNCs as compared with that of genistein (10^{-6} or 10^{-5} M) or zinc (10^{-5} or 10^{-4} M) alone.

Effect of Genistein and Zinc on Osteoclastic Cell Death

The effects of genestein or zinc on osteoclast-like cells induced in the presence of M-CSF and

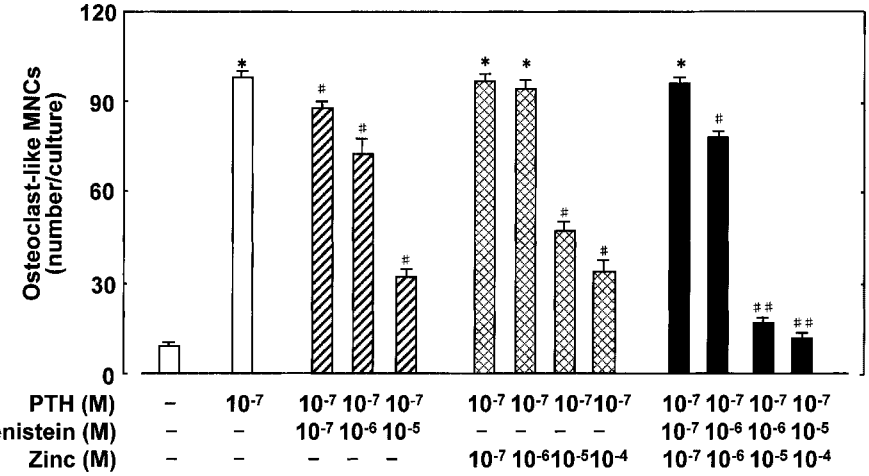

Fig. 1. Effects of genistein or zinc on PTH-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, genistein (10^{-7}–10^{-5} M), zinc (10^{-7}–10^{-4} M), or genistein (10^{-7}–10^{-5} M) plus zinc (10^{-7}–10^{-4} M) in the presence or absence of PTH (10^{-7} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. $^{*}P < 0.01$ compared with the control (none) value. $^{\#}P < 0.01$ compared with the value for PTH alone. $^{\#\#}P < 0.01$ compared with the value for genistein (10^{-6} or 10^{-5} M H) or zinc (10^{-5} or 10^{-4} M).

RANKL in the mouse marrow culture were examined. After the M-CSF (10 ng/ml)-dependent bone marrow culture, macrophages were cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days to generate mature osteoclasts, and the osteoclastic cells were further cultured in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence (Fig. 2) of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h. The formed osteoclastic cells were not significantly decreased with culture for 24 or 72 h in the presence of M-CSF and RANKL as compared with the value obtained at the zero time (Fig. 2). The number of osteoclast-like cells was significantly decreased with culture of genistein (10^{-6} M) for 24 h, and it was significantly decreased with culture of genistein (10^{-6} M) or zinc (10^{-5} M) for 72 h (Fig. 2). Culture with genistein (10^{-6} M) plus zinc (10^{-5} M) for 24 or 72 h caused a significant decrease in osteoclast-like cells as compared with the value obtained from genistein (10^{-6} M) or zinc (10^{-5} M) alone.

When osteoclast-like cells were cultured for 24 or 72 h in the absence of M-CSF and RANKL, the number of osteoclast-like cells was significantly decreased as compared with the control value obtained at zero time (Fig. 3). Culture with genistein (10^{-6} M) or zinc (10^{-5} M) for 24 or 48 h in the absence of M-CSF and RANKL did not cause a significant decrease in osteoclast-like cells as compared with the control value obtained at 24 or 72 h of culture. The number of osteoclast-like cells was significantly decreased

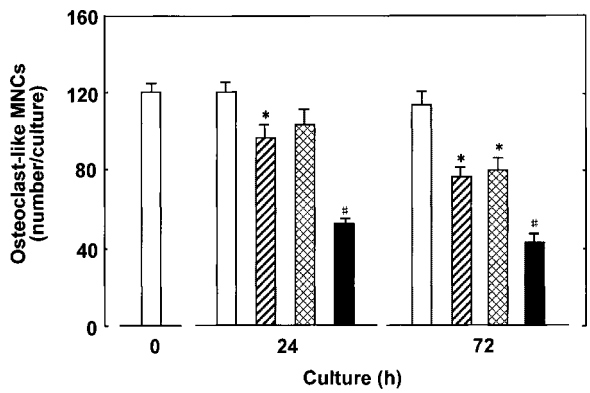

Fig. 2. Effects of genistein or zinc on osteoclastic cell death in the presence of M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the TRACP-positive MNCs formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value at zero time. $^{\#}P < 0.01$ compared with the value for genistein or zinc alone. White bars, control; hatched bars, genistein (10^{-6} M); double hatched bars, zinc (10^{-5} M); black bars, genistein (10^{-6} M) plus zinc (10^{-5} M).

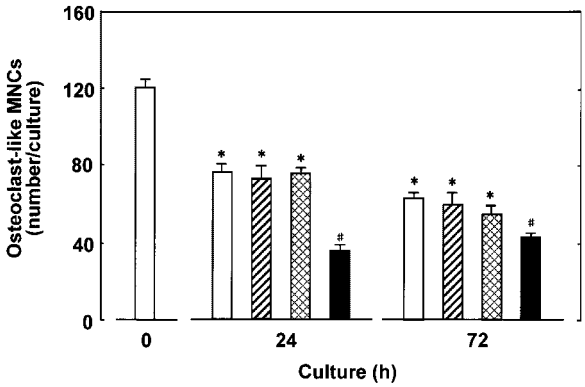

Fig. 3. Effects of genistein or zinc on osteoclastic cell death in the presence of M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the TRACP-positive MNCs formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the absence of M-CSF plus RANKL. Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value at zero time. $^{\#}P < 0.01$ compared with the control value at 24 or 72 h. White bars, control; hatched bars, genistein (10^{-6} M); double hatched bars, zinc (10^{-5} M); black bars, genistein (10^{-6} M) plus zinc (10^{-5} M).

by culture with the combination of genistein (10^{-6} M) plus zinc (10^{-5} M).

The effects of genistein or zinc on DNA fragmentation in osteoclastic cells formed with culture in the absence (Fig. 4A) or presence (Fig. 4B) of M-CSF and RANKL was examined. Osteoclastic cells were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). The adherent cells were lysed, and then the lysate was separated by electrophoresis in agarose gel. Culture with genestein plus zinc caused DNA fragmentation in the presence or absence of M-CSF and RANKL.

The effects of caspase-3 inhibitor on the genestein plus zinc-induced cell death were examined (Fig. 5). Osteoclastic cells were cultured for 24 h in medium containing either vehicle (1% ethanol) or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without caspase-3 inhibitor (10^{-8} or 10^{-7} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). The genistein plus zinc-induced decrease in osteoclastic cells was significantly inhibited with culture in the presence of caspase-3 inhibitor (10^{-8} or 10^{-7} M) as compared with the control value obtained in the presence of genestein plus zinc without caspase-3 inhibitor (Fig. 5).

Effects of Genistein and Zinc on Apoptosis-Related Gene Expression in Osteoclastic Cells

The effects of genistein or zinc on caspase-3 mRNA expression in osteoclastic cells were examined (Fig. 6A). The M-CSF-dependent bone marrow macrophages were cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days, and the osteoclastic cells were further cultured in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h. Culture with genistein (10^{-6} M) in the absence of M-CSF and RANKL for 72 h caused a significant increase in caspase-3 mRNA expression in osteoclast-like cells. Such an effect was not seen in the presence of M-CSF and RANKL. Caspase-3 mRNA expression was significantly increased with culture of zinc (10^{-5} M) in the presence or absence of M-CSF and RANKL for 24 or 72 h. The combination of genistein (10^{-6} M) plus zinc (10^{-5} M) caused a significant increase in caspase-3 mRNA expression in osteoclastic cells cultured in the presence or absence of M-CSF and RANKL for 72 h (Fig. 6A). G3PDH mRNA expression was not significantly changed in osteoclastic cells cultured in the presence of genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without M-CSF and RANKL (Fig. 6B).

The effect of cycloheximide or DRB on the genestein plus zinc-induced increase in caspase-3 mRNA expression in osteoclast-like cells was examined (Fig. 7A). The genistein (10^{-6} M) plus zinc (10^{-5} M)-induced increase in caspase-3 mRNA expression was not seen in osteoclastic cells cultured with cycloheximide (10^{-7} M), an inhibitor of protein synthesis, or DRB (10^{-6} M), an inhibitor of transcriptional activity, in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). G3PDH mRNA expression was not significantly changed in this experiment (Fig. 7B).

Effects of Genistein and Zinc on Osteoclastic Bone Resorption-Related Gene Expression

The effects of genistein or zinc on mRNA expression of TRACP or cathepsin K which is

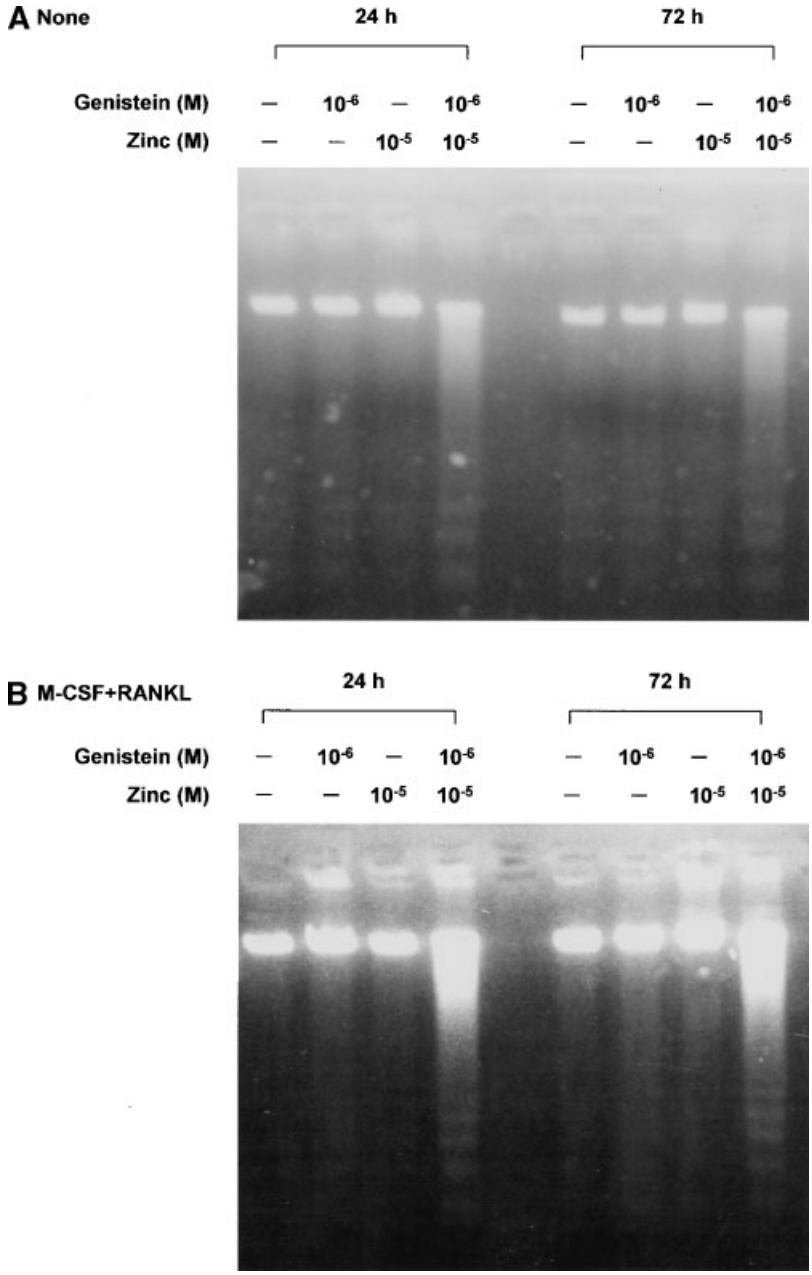

Fig. 4. Effects of genistein or zinc on DNA fragmentation in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M) in the presence (**A**) or absence (**B**) of M-CSF (10 ng/ml) plus RANKL (50 ng/ml), and the lysate (containing DNA 2 μg) of adherent cells was applied to agarose gel. The figure shows one of five experiments with separate samples.

related to osteoclastic bone resorption were examined using RT-PCR with qualitative/semi-quantitative determination (Fig. 8). The expression of TRACP mRNA was significantly decreased in osteoclastic cells cultured with genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 72 h (Fig. 8A). Genistein (10^{-6} M) or zinc (10^{-5} M) alone did not have a significant effect on TRACP mRNA expression in osteoclastic cells with or without M-CSF and RANKL.

Cathepsin K mRNA expression in osteoclastic cells was significantly decreased with culture of

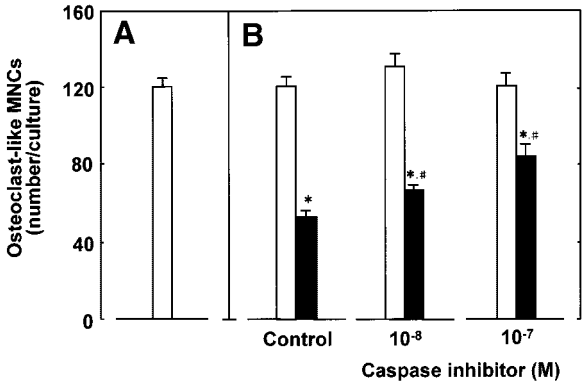

Fig. 5. Effects of genistein plus zinc in the presence of caspase-3 inhibitor on osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml) (**A**). After medium change, the osteoclast-like cells formed were cultured for 24 h in medium containing either vehicle (1% ethanol) or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without caspase-3 inhibitor (10^{-8} or 10^{-7} M) in the presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml) (**B**). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. $^{*}P < 0.01$ compared with the control (none) value with or without caspase-3 inhibitor. $^{\#}P < 0.01$ compared with the value for genistein plus zinc without caspase-3 inhibitor. White bars, without genistein plus zinc; black bars, with genistein plus zinc.

genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 72 h (Fig. 8B). The effect of genistein or zinc in decreasing cathepsin K mRNA expression was weakened in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). The combination of genistein (10^{-6} M) and zinc (10^{-5} M) caused a significant decrease in cathepsin K mRNA expression in osteoclastic cells as compared with the value for genistein or zinc alone.

G3PDH mRNA expression was not significantly changed in osteoclastic cells cultured in the presence of genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without M-CSF and RANKL, as shown in Figure 6B.

Effects of Genistein and Zinc on RANKL Signaling-Related Gene Expression in Osteoclastic Cells

The effects of genistein (10^{-6} M) or zinc (10^{-5} M) on mRNA expression of NF-kB or NFATc1, which is related to RANKL signaling, in osteoclastic cells were examined (Fig. 9). NF-kB mRNA expression in osteoclastic cells was not significantly changed with culture of genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h (Fig. 9A).

NFATc1 mRNA expression in osteoclastic cells was significantly decreased with culture of genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) as compared with the value for genistein (10^{-6} M) or zinc (10^{-5} M) alone for 24 or 72 h (Fig. 9B). Culture with zinc (10^{-5} M) caused a significant decrease in NFATc1 mRNA expression in osteoclastic cells cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 72 h as compared with the control value.

G3PDH mRNA expression was not significantly changed in osteoclastic cells cultured in the presence of genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) with or without M-CSF and RANKL, as shown in Figure 6B.

DISCUSSION

Genistein or zinc has been shown to have an inhibitory effect on various bone-resorbing factors-induced bone resorption in rat femoral tissues in vitro [Yamaguchi et al., 1992; Yamaguchi and Gao, 1998a]. Genistein or zinc has a suppressive effect on osteoclastogenesis in mouse marrow culture in vitro [Kishi and Yamaguchi, 1994; Gao and Yamaguchi, 1999b]. The effect of genistein or zinc on mature osteoclasts, however, has not been clarified. This study was undertaken to determine whether genistein or zinc has a suppressive effect on osteoclastic cells in mouse marrow culture. We found that the combination of genistein and zinc has a potent suppressive effect on osteoclastic cells generated in mouse marrow culture in vitro.

Culture with PTH markedly stimulated the formation of osteoclast-like cells in mouse marrow culture. This stimulation was significantly inhibited in the presence of genistein or zinc. The combination of genistein and zinc was found to have a potent suppressive effect on osteoclastogenesis as compared with the effect of each factor alone. The additive effect was seen with the combination of genistein (10^{-6} M) plus zinc (10^{-5} M) or genistein (10^{-5} M) plus zinc (10^{-4} M). The effect of two combinations with

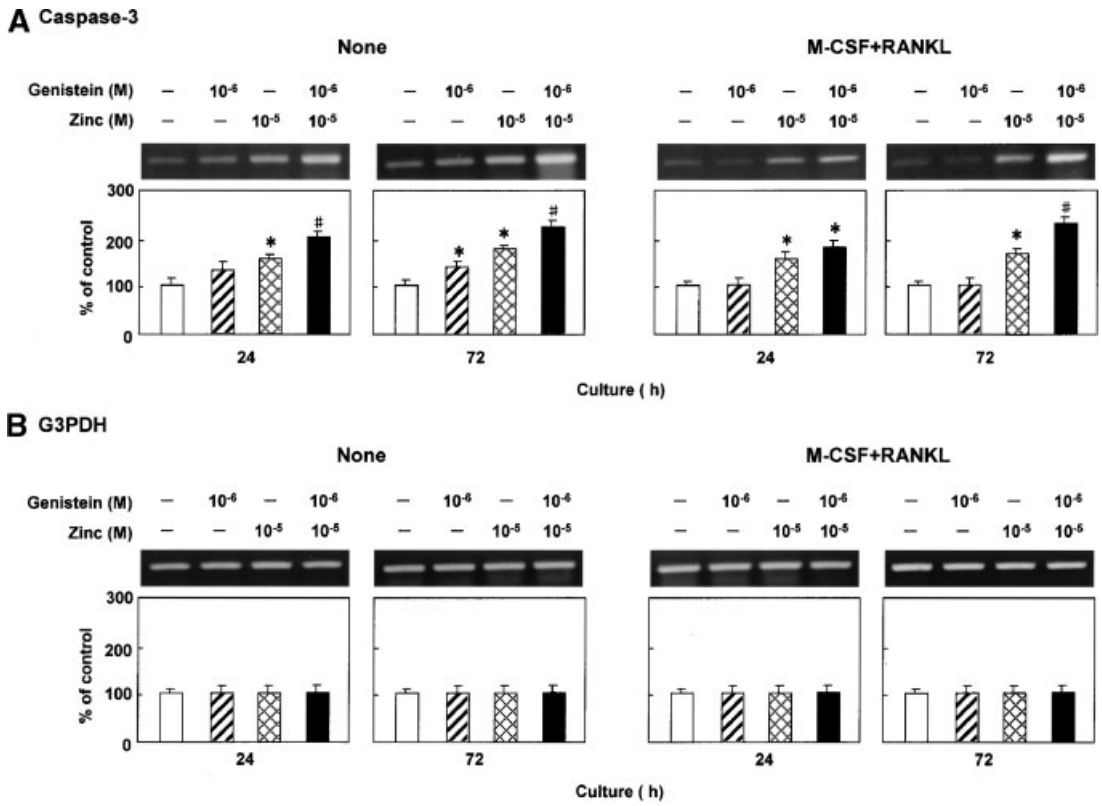

Fig. 6. (**A**) Effects of genistein or zinc on mRNA expression of caspase-3 and G3PDH (**B**) in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (2 μg) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as % of control (mean ± SEM of five experiments). $^{*}P < 0.01$ compared with control (none) value.

different concentration was saturated with the used concentration.

The number of mature osteoclastic cells, which were generated in mouse marrow culture with M-CSF and RANKL, was significantly decreases with culture of genistein (10^{-6} M) plus zinc (10^{-5} M) as compared with the value for each factor alone. The combination of genistein and zinc had a potent suppressive effect on mature osteoclastic cells. Such an effect was remarkable in the presence of M-CSF and RANKL, although it was also seen in the absence of M-CSF and RANKL. It is speculated that the combination of genestein and zinc has an inhibitory effect on RANKL signaling-dependent pathways in osteoclastic cells.

Culture with genistein and zinc caused DNA fragmentation in osteoclastic cells, indicating that the factors stimulate apoptotic cell death. In addition, the genistein plus zinc-induced cell death was significantly prevented in the presence of caspase-3 inhibitor. However, this effect was partial. Presumably, the stimulation of apoptotic cell death with the combination of genistein and zinc is partly mediated through activation of caspase-3, and also other mechanisms participate in apoptotic cell death.

Zinc was found to increase the expression of caspase-3 mRNA in osteoclastic cells cultured with or without M-CSF and RANKL. This effect was significantly enhanced with the combination of genistein. However, genistein did not have the effect in the presence of M-CSF and RANKL. This finding may support the view that the combination of genistein and zinc has a potent stimulatory effect on apoptotic cell death which is partly mediated through caspase-3 in osteoclastic cells.

The stimulatory effect with the combination of genistein and zinc on caspase-3 mRNA

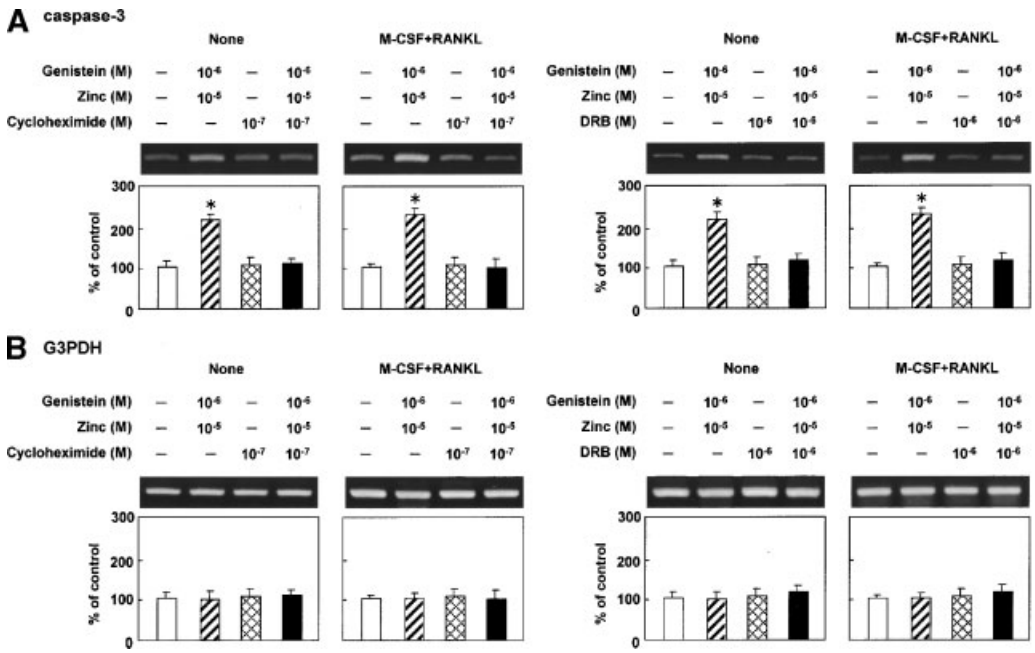

Fig. 7. Effects of cycloheximide or DRB on the genistein plus zinc stimulated caspase-3 (**A**) as G3PDH (**B**) mRNA expression in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M) plus zinc (10^{-5} M) with or without cycloheximide (10^{-7} M) or DRB (10^{-6} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (2 μg) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as % of control (mean ± SEM of five experiments). $^*P < 0.01$ compared with the control (none) value.

expression was completely inhibited with culture of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcriptional activity, in osteoclastic cells with and without M-CSF and RANKL. This result suggests that the effect with the combination of genistein and zinc on caspase-3 mRNA expression is involved in protein synthesis and transcription activation in osteoclastic cells. Genistein or zinc has been demonstrated to have a stimulatory effect on protein synthesis due to activating aminoacyl-tRNA synthesis, a rate-limiting enzyme in protein synthesis, in osteoclastic cells [Yamaguchi et al., 1994; Yamaguchi and Sugimoto, 2000].

The activation of TRACP or cathepsin K plays a role in the promotion of osteoclastic bone resorption [Zaidi et al., 2003]. Culture with genistein or zinc for 72 h was found to induce a significant decrease in cathepsin K mRNA expression in osteoclastic cells cultured in the presence or absence of M-CSF and RANKL. The combination of genistein and zinc had a potent suppressive effect on cathepsin K mRNA expression as compared with the effect of each factor. The expression of TRACP mRNA was significantly decreased in the presence of genistein plus zinc in osteoclastic cells cultured with M-CSF and RANKL for 72 h, although the method with RT-PCR is qualitative/semi-quantitative determination. Genistein or zinc alone did not have a significant effect on TRACP mRNA in osteoclastic cells. These results suggest that the combination of genistein and zinc has suppressive effects on the activity of cathepsin K or TRACP in osteoclastic cells which are activated through RANKL signaling. It is speculated that the combination of genistein and zinc has potent suppressive effect on bone-resorbing activity. The examination with Western blot analysis, however, remains to be elucidated.

The RANK activation by binding of RANKL stimulates the NF-kB signaling, probably through association with TRAF-6 in osteoclasts [Darnay et al., 1998, 1999]. RANKL signaling targets include mitogen-activated protein kinases (MAPKs), NF-kB, and NFATc1 in osteoclast differentiation [Kim et al., 2005]. Culture with the combination of genistein and

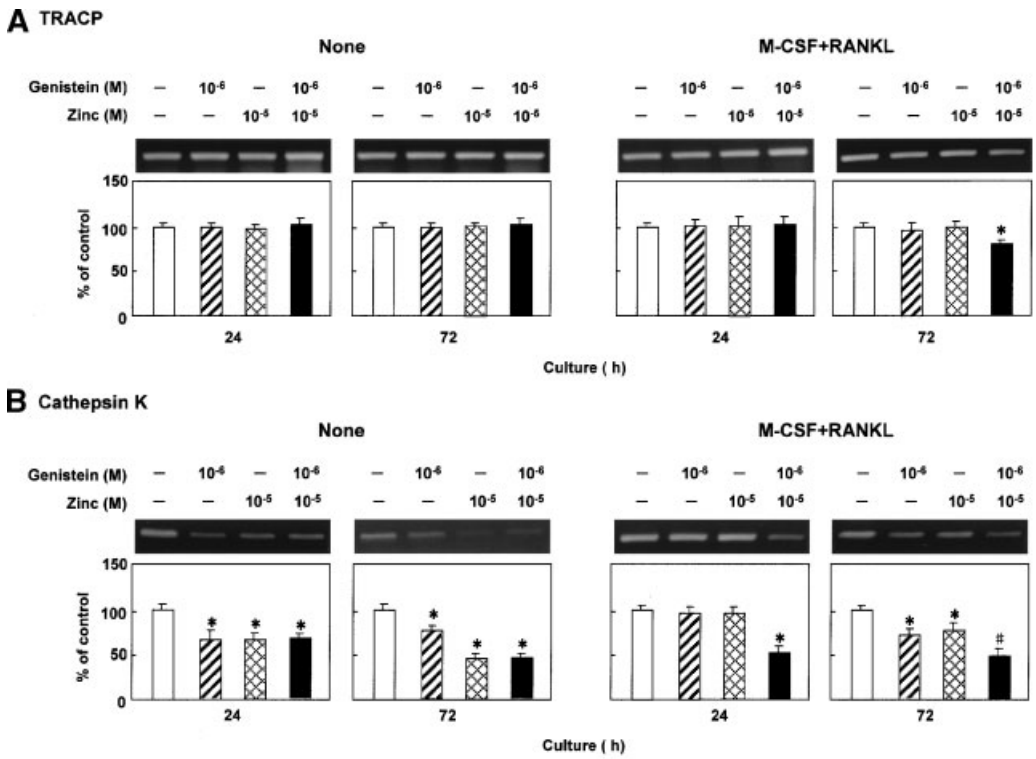

Fig. 8. Effects of genistein or zinc on mRNA expression of TRACP (**A**) or cathepsin K (**B**) in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in the Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (2 μg) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as % of control (mean ± SEM of five experiments). $^{*}P < 0.01$ compared with the control (none) value. $^{\#}P < 0.01$ compared with the value for genistein or zinc alone.

zinc did not cause a significant change in NF-kB mRNA expression in osteoclastic cells in the presence or absence of M-CSF and RANKL. NFATc1 mRNA expression was significantly increased with culture of the combination of genistein and zinc in osteoclastic cells cultured in the presence of M-CSF and RANKL. Genistein and zinc may affect on the gene expression of NFATc1 that is a target protein of RANKL signaling in osteoclastic cells. It is speculated that the decrease in NFATc1 expression with the combination of genistein and zinc is important in the revelation of suppressive effect on osteoclastogenesis and osteoclastic cell function.

The combination of genistein and zinc was found to have additive or synergistic effects in the suppression of PTH-induced osteoclastogenesis, mature osteoclastic cell death, and bone resorption-related gene expression in osteoclastic cells in the presence of M-CSF and RANKL. Genistein or zinc has been shown to inhibit bone-resorbing factors-induced bone resorption in bone tissue cultures in vitro and osteoclastogenesis in bone marrow cultures in vitro [Yamaguchi et al., 1992; Kishi and Yamaguchi, 1994; Yamaguchi and Gao, 1998b; Gao and Yamaguchi, 1999b]. The cellular mechanism by which genistein or zinc has inhibitory effects on osteoclastic bone resorption is different [Yamaguchi, 1998, 2002]. The combination of each factor which the mechanism of their actions is difference may have synergistic effects on osteoclastic cells. In addition, the combination of genistein and zinc has been demonstrated to have synergistic effects on osteoblastic MC3T3-E1 cells [Uchiyama and Yamaguchi, 2006]. The combination of genistein and zinc has a role in the prevention of bone loss with increasing age.

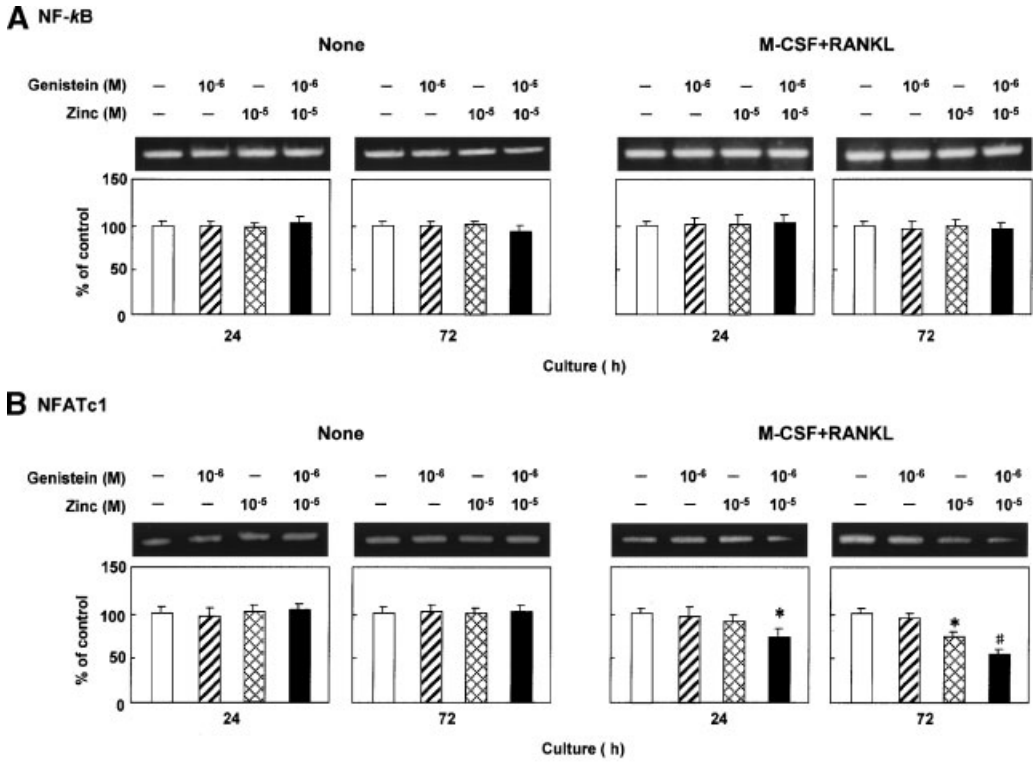

Fig. 9. Effects of genistein or zinc on mRNA expression of NF-kB (**A**) or NFATc1 (**B**) in osteoclastic cells formed in mouse marrow culture. Mouse marrow cells were cultured as described in Materials and Methods. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the osteoclast-like cells formed were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), genistein (10^{-6} M), zinc (10^{-5} M), or genistein (10^{-6} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). Total RNAs (2 μg) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as % of control (mean ± SEM of five experiments). $^{*}P < 0.01$ compared with the control (none) value. $^{\#}P < 0.01$ compared with the value for genistein or zinc alone.

In conclusion, it has been demonstrated that genistein and zinc synergistically stimulate apoptotic cell death and suppress RANKL signaling-related gene expression in osteoclastic cells in vitro.

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