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Inhibitory Effect of Genistein on Osteoclast-Like Cell Formation in Mouse Marrow Cultures

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ABSTRACT. The effect of genistein on osteoclast-like cell formation in mouse marrow culture *in vitro* was investigated. The bone marrow cells were cultured for 7 days in α-minimal essential medium containing a well-known bone resorbing agent [parathyroid hormone (1–34) (PTH), prostaglandin E_2 (PGE_2), 1,25-dihydroxyvitamin D_3 (VD_3), or lipopolysaccharide (LPS)] with an effective concentration. Osteoclast-like cell formation was estimated by staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts. The presence of PTH (10^{-8} M), PGE_2 (10^{-6} M), VD_3 (10^{-8} M), or LPS (1 μg/mL) induced a remarkable increase in osteoclast-like multinucleated cells. These increases were inhibited significantly in the presence of genistein (10^{-7} to 10^{-5} M). The inhibitory effect of genistein (10^{-5} M) was equal to that of 17 β-estradiol (10^{-8} M), calcitonin (10^{-9} M), or zinc sulfate (10^{-5} M). Genistein (10^{-5} M) significantly inhibited dibutyryl cyclic adenosine monophosphate (10^{-5} M)-induced osteoclast-like cell formation. However, genistein (10^{-5} M) did not inhibit phorbol 12-myristate 13-acetate-induced osteoclast-like cell formation. The present study demonstrated that genistein has a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture. The inhibitory action of genistein may involve in cyclic AMP signaling. BIOCHEM PHARMACOL **58**;5:767–772, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. genistein; daidzein; estrogen; bone resorption; osteoclastic formation; bone marrow culture

Osteoporosis is widely recognized as a major public health problem [1, 2]. Nutritional and pharmacological factors are needed to prevent bone loss with increasing age. The chemical compounds that act on bone metabolism as nutrients in food, however, are poorly understood. Genistein is a natural isoflavonoid phytoestrogen found in *Leguminosae*. This isoflavonoid has been shown to have a strong inhibitory effect on protein tyrosine kinases [3, 4], and it can produce cell cycle arrest and apoptosis in leukemic cells [4, 5]. The mechanism of action of genistein, however, has not been fully clarified.

It has been shown recently that genistein may influence bone metabolism [6, 7]. Blair *et al.* [6] reported that genistein inhibits avian osteoclastic activity and can reduce bone loss in ovariectomized rats. Dietary soybean protein has been shown to prevent bone loss in ovariectomized rats [7]. Moreover, it has been found that genistein has an anabolic effect on bone formation and mineralization by cultured bone cells [8–10]. More recently, genistein has been demonstrated to inhibit bone resorption in femoral-metaphyseal tissues obtained from elderly rats *in vitro* [11]. Thus, genistein may have a direct anabolic effect on bone metabolism, suggesting a possible role in the prevention of osteoporosis.

The present study was undertaken to clarify the cellular mechanism of genistein inhibition of bone resorption. We examined the effect of genistein on osteoclast-like cell formation in a mouse marrow culture system *in vitro*. It was found that genistein has an inhibitory effect on osteoclast-like cell formation induced by well-known bone resorbing agents.

MATERIALS AND METHODS

Chemicals

α-MEM† and penicillin-streptomycin (5000 U/mL of penicillin; 5000 μg/mL of streptomycin) were obtained from Gibco Laboratories. Fetal bovine serum was obtained from Bioproducts Inc. Genistein, daidzein, VD_3, PGE_2, LPS, 17 β-estradiol, PMA, and DcAMP were obtained from the Sigma Chemical Co. Synthetic human PTH (1–34) and synthetic [$Asu^{1,7}$] eel calcitonin were supplied by the Asahi Chemical Industry Co., Ltd. Zinc sulfate and other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries. All water used was glass-distilled.

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† *Abbreviations:* αMEM, α-minimal essential medium; PTH, parathyroid hormone (1–34); PGE_2, prostaglandin E_2; VD_3, 1,25-dihydroxy vitamin D_3; LPS, lipopolysaccharide; TRACP, tartrate-resistant acid phosphatase; MNCs, multinucleated cells; PMA, phorbol 12-myristate 13-acetate; and DcAMP, dibutyryl cyclic adenosine monophosphate.

Animals

Male mice (ddY strain; 6 weeks old) were obtained from Japan SLC. 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 exsanguination.

Marrow Cultures

Bone marrow cells were isolated from mice, as reported elsewhere [12, 13]. 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% heat-inactivated fetal bovine serum 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°. 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 were added to the culture medium containing either vehicle, PTH (10^{-8} M), PGE_2 (10^{-6} M), VD_3 (10^{-8} M), or LPS (1 μg/mL) with an effective concentration at the beginning of the cultures and at the time of medium change. In separate experiments, the respective media contained either calcitonin, 17 β-estradiol, zinc sulfate, PMA, or DcAMP.

Enzyme Histochemistry

After being cultured for 7 days, cells adherent to the 24-well plates were stained for TRACP, a marker enzyme of osteoclasts [14, 15]. 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 [14]. The fixed cells were incubated for 12 min at room temperature (25°) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate, and red violet LB salt (Sigma) as a stain for the reaction product, in the presence of 10 mM sodium tartrate [14]. TRACP-positive MNCs containing three or more nuclei were counted as osteoclast-like cells.

Pit Formation Assay

The pit formation assay was performed according to the method of Takada *et al.* [16] with some modifications. Briefly, transverse slices of dentine (150 μm in thickness) were prepared using a low-speed diamond saw (Leitz). Each slice was ground to 100 μm in thickness and sterilized in 70% ethanol overnight. For this assay, marrow cells were cultured on dentine slices for 7 days in the presence of PTH (10^{-8} M) or PGE_2 (10^{-6} M). After incubation, the slices were examined for TRACP staining. After counting TRACP-positive MNCs on a slice, the slice was subjected to ultrasonication to remove attached cells and subsequently stained with toluidine blue (0.1%, w/v). The number of pits formed on the slices was determined using a light microscope.

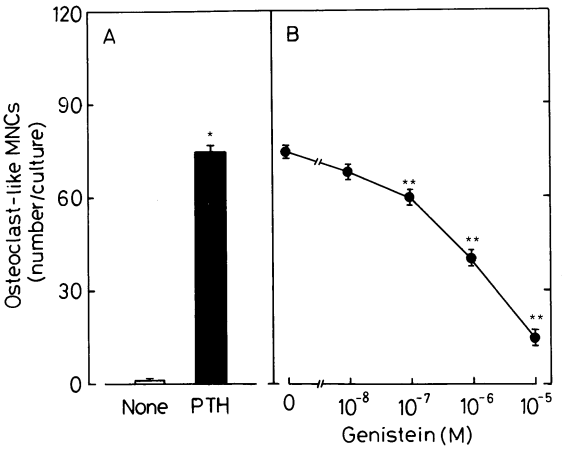

FIG. 1. Effect of genistein on PTH-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-8} M), or PTH (10^{-8} M) plus genistein (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value; and (**) $P < 0.01$, compared with the value for PTH alone. Panel A: none (□) or PTH (■). Panel B: PTH plus genistein.

Statistical Methods

Data are expressed as means ± SEM. Statistical differences were analyzed using Student's paired *t*-test. A *P* value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

The effect of genistein on the bone-resorbing factor-induced osteoclast-like MNC formation in the mouse marrow culture system was examined. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-8} M), PGE_2 (10^{-6} M), VD_3 (10^{-8} M), or LPS (1 μg/mL of medium) in the absence or presence of genistein (10^{-8} to 10^{-5} M). The number of TRACP-positive MNCs was increased significantly in the presence of PTH (Fig. 1A), PGE_2 (Fig. 2A), VD_3 (Fig. 3A), or LPS (Fig. 4A). TRACP-positive MNCs were not formed appreciably in the control culture without bone-resorbing factors at any incubation time. The presence of genistein (10^{-7} to 10^{-5} M) in the culture medium caused a significant decrease in the number of TRACP-positive MNCs stimulated by PTH (Fig. 1B), PGE_2 (Fig. 2B), VD_3 (Fig. 3B), or LPS (Fig. 4B). Genistein in the range of 10^{-8} to 10^{-5} M did not have an inhibitory effect on the proliferation of marrow cells; this was independent of the presence of bone-resorbing factors (data not shown).

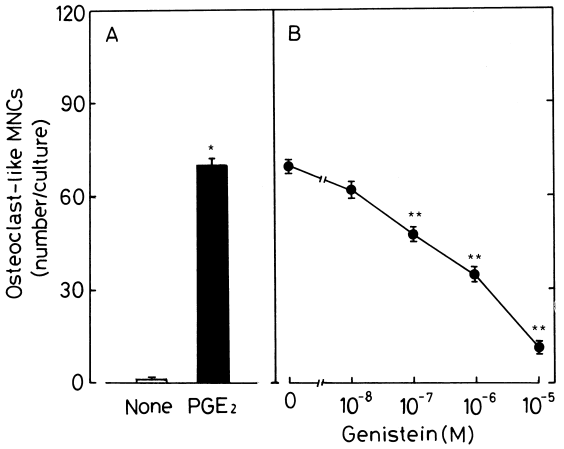

FIG. 2. Effect of genistein on PGE_2-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PGE_2 (10^{-6} M), or PGE_2 (10^{-6} M) plus genistein (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value; and (**) $P < 0.01$, compared with the value for PGE_2 alone. Panel A: none (□) or PGE_2 (■). Panel B: PGE_2 plus genistein.

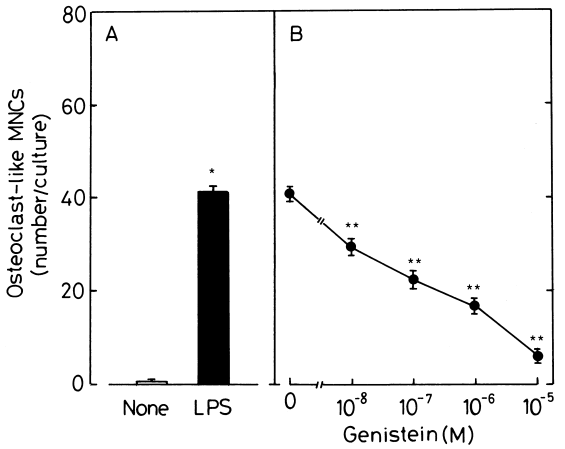

FIG. 4. Effect of genistein on LPS-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, LPS (1 μg/mL), or LPS (1 μg/mL) plus genistein (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value; and (**) $P < 0.01$, compared with the value for LPS alone. Panel A: none (□) or LPS (■). Panel B: LPS plus genistein.

The effect of daidzein on PTH- or PGE_2-induced osteoclast-like MNC formation in the mouse marrow culture is shown in Fig. 5. The presence of daidzein (10^{-5} M) in the culture medium caused a significant decrease in the number of TRACP-positive MNCs stimulated by PTH (10^{-8} M) or PGE_2 (10^{-6} M). Daidzein at 10^{-6} M had no effect on the PTH- or PGE_2-induced osteoclast-like MNC formation.

The effects of genistein and other agents on PTH- or PGE_2-induced osteoclast-like MNC formation in mouse marrow culture were compared (Table 1). Mouse marrow cells were cultured for 7 days in medium containing either vehicle, genistein, 17 β-estradiol, calcitonin, or zinc sulfate at the indicated concentration. Each caused a significant

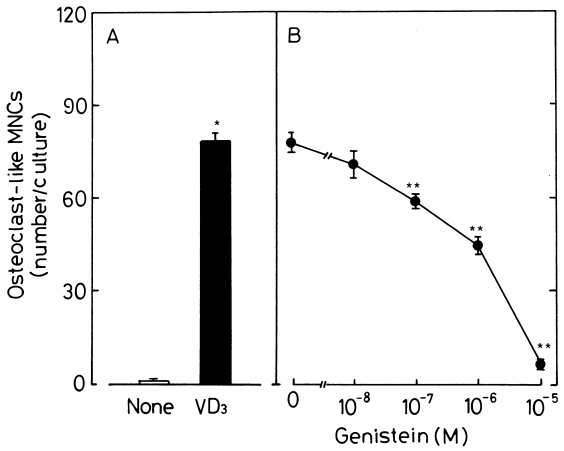

FIG. 3. Effect of genistein on VD_3-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, VD_3 (10^{-8} M), or VD_3 (10^{-8} M) plus genistein (10^{-8} to 10^{-5} M). Cells then were fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value; and (**) $P < 0.01$, compared with the value for VD_3 alone. Panel A: none (□) or VD_3 (■). Panel B: VD_3 plus genistein.

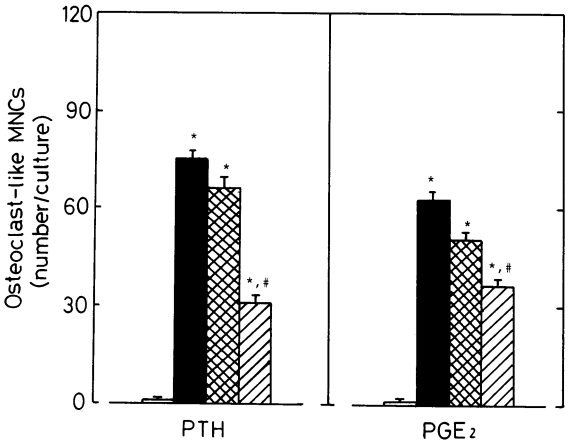

FIG. 5. Effect of daidzein on PTH- or PGE_2-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-8} M), PTH (10^{-8} M) plus daidzein (10^{-6} and 10^{-5} M), PGE_2 (10^{-6} M), or PGE_2 (10^{-6} M) plus daidzein (10^{-6} and 10^{-5} M). Cells then were fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value; and (#) $P < 0.01$, compared with the value for PTH or PGE_2 alone. Columns: none (□), PTH or PGE_2 alone (■), PTH or PGE_2 plus daidzein (10^{-6} M) (▨), or PTH or PGE_2 plus daidzein (10^{-5} M) (▨).

TABLE 1. Comparision of the effects of genistein and other agents on the PTH- or PGE_2-induced osteoclast-like cell formation in mouse marrow culture

Treatment	Osteoclast-like MNCs (number/culture)	
	PTH	PGE_2
Control	79.6 ± 3.5	62.3 ± 3.1
Genistein (10^{-5} M)	16.5 ± 0.8*	17.3 ± 0.6*
17 β-Estradiol (10^{-8} M)	14.5 ± 3.9*	23.3 ± 1.5*
Calcitonin (10^{-9} M)	12.8 ± 1.7*	15.3 ± 1.1*
Zinc sulfate (10^{-5} M)	9.7 ± 0.2*	19.0 ± 1.4*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-8} M), PGE_2 (10^{-6} M), or a bone-resorbing agent. Each value is the mean ± SEM of five cultures.

* $P < 0.01$, compared with the control value of PTH or PGE_2 alone.

inhibition of osteoclast-like MNC formation induced by PTH or PGE_2.

In another experiment, mouse marrow cells were cultured for 3 days in medium containing either vehicle, PTH (10^{-8} M), or PGE_2 (10^{-6} M) in the presence of genistein (10^{-6} and 10^{-5} M) or 17 β-estradiol (10^{-8} M); then genistein or 17 β-estradiol was removed from the culture medium containing each bone-resorbing agent, and the cells were further incubated for 4 days. In this case, the presence of genistein caused a slight inhibition of osteoclast-like MNC formation induced by all bone-resorbing agents (Table 2). However, 17 β-estradiol had a potent inhibitory effect. In still another experiment, mouse marrow cells were cultured for 3 days in medium containing either vehicle, PTH (10^{-8} M), or PGE_2 (10^{-6} M); then genistein (10^{-6} and 10^{-5} M) was added to the culture medium containing each bone-resorbing agent, and the cells were further incubated for 4 days. The inhibitory effect

TABLE 2. Effect of genistein or 17 β-estradiol on the bone-resorbing agent-induced osteoclast-like cell formation in mouse marrow culture

Treatment	Osteoclast-like MNCs (number/culture)	
	PTH	PGE_2
Earlier stage		
Control	77.5 ± 2.1	63.8 ± 4.7
Genistein (10^{-6} M)	65.3 ± 2.1*	51.0 ± 2.0*
Genistein (10^{-5} M)	63.3 ± 1.9*	38.5 ± 2.0*
17 β-Estradiol (10^{-8} M)	32.0 ± 2.1*	20.8 ± 1.3*
Later stage		
Control	75.3 ± 1.5	65.3 ± 3.4
Genistein (10^{-6} M)	56.0 ± 3.8*	36.8 ± 2.1*
Genistein (10^{-5} M)	22.8 ± 1.6*	10.3 ± 0.4*
17 β-Estradiol (10^{-8} M)	21.0 ± 1.2*	31.0 ± 1.1*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PTH (10^{-8} M), or PGE_2 (10^{-6} M). After 3 days of culture (earlier stage), the medium was changed, and the cells were cultured for an additional 4 days (later stage). The cells were cultured at either the earlier stage or the later stage in the presence of genistein or 17 β-estradiol. Each value is the mean ± SEM of five cultures.

*$P < 0.01$, compared with the control value of PTH or PGE_2 alone.

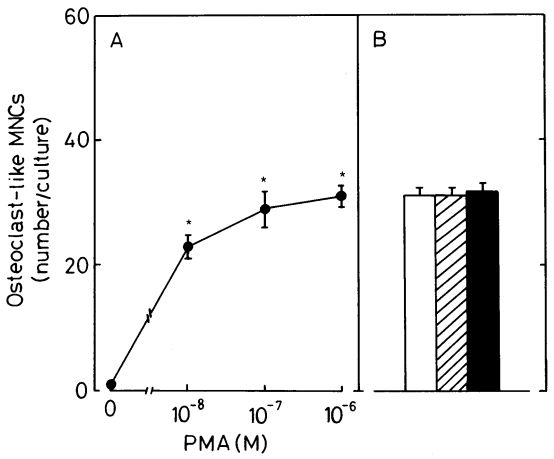

FIG. 6. Effect of genistein on PMA-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PMA (10^{-8} to 10^{-6} M), or PMA (10^{-6} M) plus genistein (10^{-6} and 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none) value. Panel A: PMA (10^{-8} to 10^{-6} M) alone. Panel B: PMA (10^{-6} M) (□), PMA (10^{-6} M) plus genistein (10^{-6} M) (▨), or PMA (10^{-6} M) plus genistein (10^{-5} M) (■).

of genistein at 10^{-5} M was equal to that of 17 β-estradiol at 10^{-8} M (Table 2).

The effect of PMA on osteoclast-like MNC formation in mouse marrow cultures is shown in Fig. 6. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, PMA (10^{-8} to 10^{-6} M), or PMA (10^{-6} M) plus genistein (10^{-6} and 10^{-5} M). The presence of PMA (10^{-8} to 10^{-6} M) in medium caused a significant increase in osteoclast-like MNC formation. The effect of PMA was saturated at 10^{-6} M (Fig. 6A). This increase was not altered significantly by the addition of genistein (10^{-6} or 10^{-5} M) (Fig. 6B).

The effect of DcAMP on osteoclast-like MNC formation in mouse marrow culture is shown in Fig. 7. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, DcAMP (10^{-7} to 10^{-5} M) or DcAMP (10^{-5} M) plus genistein (10^{-6} or 10^{-5} M). Osteoclast-like cell formation was elevated significantly in the presence of DcAMP (10^{-7} to 10^{-5} M) (Fig. 7A). The effect of DcAMP at 10^{-5} M was remarkable. This increase was inhibited significantly by the addition of genistein (10^{-5} M) (Fig. 7B).

We also cultured unfractionated bone marrow cells on a dentine slice and examined the effect of genistein on the number of resorption pits formed over 7 days. Genistein (10^{-5} M) significantly inhibited the PTH (10^{-8} M) or PGE_2 (10^{-6} M)-induced increase in the number of pits formed on a dentine slice; the number decreased from 68 ± 7 to 25 ± 4 or from 51 ± 6 to 18 ± 4 (mean ± SEM of five slices), respectively.

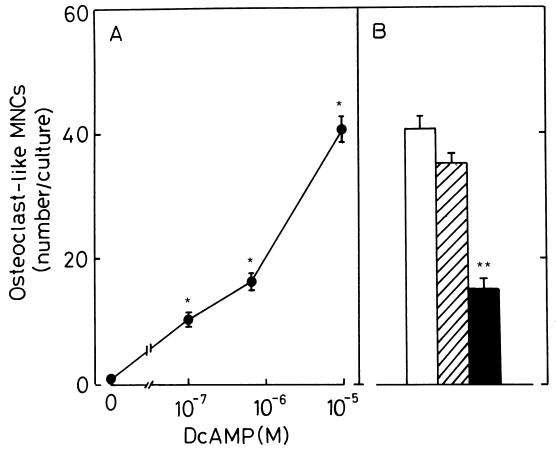

FIG. 7. Effect of genistein on DcAMP-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle, DcAMP (10^{-7} to 10^{-5} M), or DcAMP (10^{-5} M) plus genistein (10^{-6} or 10^{-5} M). Cells then were fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of five cultures. Key: (*) $P < 0.01$, compared with the control (none); and (**) $P < 0.01$, compared with the value for DcAMP (10^{-5} M) alone. Panel A: DcAMP (10^{-7} to 10^{-5} M). Panel B: DcAMP (10^{-5} M) (□), DcAMP (10^{-5} M) plus genistein (10^{-6} M) (▨), or DcAMP (10^{-5} M) plus genistein (10^{-5} M) (■).

DISCUSSION

It is known that PTH, PGE_2, VD_3, and LPS stimulate osteoclastic bone resorption *in vitro* [17–20]. The presence of PTH (10^{-8} M), PGE_2 (10^{-6} M), VD_3 (10^{-8} M), or LPS (1 μg/mL) clearly increased the formation of osteoclast-like TRACP-positive MNCs from mouse marrow cells *in vitro*, confirming previous studies [11, 20]. The effect of bone-resorbing factors in increasing osteoclast-like MNC formation was inhibited markedly in the presence of genistein (10^{-7} to 10^{-5} M). The inhibitory effect of genistein was equal to the effect of other anti-bone-resorbing agents (calcitonin, 17 β-estradiol, and zinc sulfate) [21] on osteoclast-like cell formation in mouse marrow culture. Thus, it was found that genistein had a potent inhibitory effect on osteoclast-like MNC formation in mouse marrow culture. At present, the mechanism by which osteoclast-like cell formation is decreased by genistein is unknown. And the possibility that genistein may decrease the proliferation and/or differentiation of cells in the pre-osteoclast subpopulation should not be ruled out.

The inhibitory effect of genistein on osteoclast-like MNC formation in mouse marrow culture seemed to be more potent than that of daidzein, which is a kind of isoflavone. Genistein revealed a more potent inhibitory effect at the later stage of differentiation of marrow cells. Genistein and daidzein are phytoestrogens. Genistein has been reported to bind the estrogen receptor (ERβ) with a high affinity and low capacity for 17 β-estradiol on bone cells [22]. The binding affinity of genistein to the receptors is lower than that of estrogen [22]. Therefore, it is possible that genistein binds to the estrogen receptor, and that the isoflavone may be exerting effects similar to those of estrogen, which can inhibit osteoclast-like cell formation.

Genistein significantly inhibited DcAMP-induced osteoclast-like MNC formation, whereas the isoflavonoid did not have an inhibitory effect on PMA-induced osteoclast-like cell formation. PMA can directly activate protein kinase C [23]. These results suggest that genistein can inhibit osteoclast-like MNC formation stimulated by the cyclic AMP signaling-dependent pathway, but not by protein kinase C signaling. The stimulatory effect of PTH or PGE_2 on osteoclast-like cell formation from mouse marrow cells has been shown to be mediated through the cyclic AMP signaling pathway [24, 25]. Presumably, an inhibitory effect of genistein on osteoclast-like MNC formation induced by PTH or PGE_2 is partly based on the blocking action for the pathway of cyclic AMP signaling at the differentiation stage of marrow cells.

It has been reported that genistein can inhibit tyrosine kinase [4]. However, it is unknown whether genistein directly inhibits cyclic AMP-dependent kinase (A kinase) in mouse marrow cell culture. More recently, it has been shown that genistein directly induces cardiac cystic fibrosis transmembrane regulator chloride current by a tyrosine kinase-independent and protein kinase A-independent pathway in guinea pig ventricular myocytes [26]. It is possible, however, that genistein may inhibit protein kinase A directly at the differentiation stage of marrow cells. In addition, if genistein can inhibit tyrosine kinase [4], the action of the isoflavone on osteoclastic cell formation may be related, in part, to an inhibitory effect on the enzyme.

Moreover, genistein had an inhibitory effect on VD_3-induced osteoclast-like cell formation from marrow cells. The mechanism by which genistein inhibits the effect of VD_3 may be different from those mechanisms causing its inhibitory action on protein kinase A and tyrosine kinase in differentiation of bone marrow cells. This remains to be elucidated.

Genistein has been demonstrated to stimulate bone mineralization in a tissue culture system *in vitro* [9, 10]. Previous studies have shown that genistein could inhibit bone resorption induced by bone-resorbing agents in tissue culture [11]. The present study clearly demonstrates that genistein can inhibit osteoclast-like cell formation in mouse marrow culture. Thus, genistein may be a useful tool in the prevention of and therapy for osteoporosis.

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