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The Effects of Hesperidin on the Proliferation and Activity of Bone Cells

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The importance of phytoestrogens to human health is currently being actively investigated. Hesperidin, abundantly found in citrus fruits, is known to possess antioxidant, anticancer, and anti-inflammatory effects. Recently, it has been reported that hesperidin inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. In our study, to determine the possible role of hesperidin in the regulation of bone metabolism, we observed the effects of hesperidin on the proliferation and activity of osteoblasts, as well as the effects of hesperidin on osteoclast generation and activity. We observed that, when treated with hesperidin, the number and viability of osteoblastic cells increased, alkaline phosphatase (ALP) activity of osteoblastic cells increased, and osteoprotegerin (OPG) secretion from MG63 cells decreased. Hesperidin treatment had no effect on the osteoclast generation and activity in the bone marrow cell culture, but decreased the number and resorptive activity of osteoclasts generated from RAW264.7 cells. Taken together, these results indicate that hesperidin increases the proliferation and activity of osteoblasts, while inhibiting generation and activity of osteoclasts. Although the precise role of hesperidin remains to be elucidated, our study suggests that it is one of the important modulators of bone metabolism.

Keywords: hesperidin, osteoclast, osteoblast, osteoprotegerin

Introduction

Osteoporosis is the most common bone disease, characterized by reduced bone mineral density and an increased risk of fracture (Adlercreutz *et al.*, 1992). Particularly, in postmenopausal women, osteoporosis is one of the critical disorders involving high bone turnover and bone loss attributed to estrogen deficiency (Heaney *et al.*, 1978). Estrogen replacement therapy, recommended only for women who are at high risk for osteoporosis, seems to be the most effective method to reduce the rate of postmenopausal bone loss, but it can be accompanied by numerous adverse effects such as uterine bleeding and increased risk of cancer (Genant *et al.*, 1989; Recker, 1993). Other treatment approaches have also shown numerous side effects (Recker, 1993; Rodan, 1994).

Although osteoporosis is mainly associated with estrogen deficiency after menopause, it is well established that environmental factors also contribute to the risk of osteoporosis. Nutrition research has shown that the human diet contains, in addition to essential macro- and micro-nutrients, a complex array of naturally occurring bioactive molecules called phytochemicals which may confer health benefits (Setchell, 1998). The consequence of exposure to environmental estrogens had been well studied, beginning more than half a century ago (Bennetts *et al.*, 1946); however, there is scant literature concerning the effects of other polyphenols on bone health.

There is some evidence that nonsteroidal, estrogen-like compounds, such as phytoestrogens, can prevent bone loss in osteoporotic animal models and in postmenopausal women (Ishimi *et al.*, 1990; Anderson *et al.*, 1995; Arjmandi *et al.*, 1996). Chemically, the phytoestrogens can be divided into the following three classes: flavonoids (genistein,

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narigenine, hesperidin, and kaempferol); coumestans (such as coumestrol); and lignans (such as enterodiol and enterolactone). Phytoestrogens are weak estrogens and can compete with estrogen for binding to the estrogen receptor and, thus, block the mitogenic effects of estrogen, while conferring cardioprotective and antiosteoporotic benefits.

Many recent studies suggest that flavonoids might also exert a protective effect against osteopenia and osteoporosis. It has been reported that certain vegetables, such as onion and Italian parsley, can prevent bone resorption in ovariectomized rats (Muhlbauer and Li, 1999). Onion extracts, which inhibit bone resorption *in vitro*, prevent cancellous and cortical bone loss induced by a combination of low protein intake and diet-mediated, mild hyperparathyroidism in rats (Ingold *et al.*, 1998).

Citrus fruits contain various bioflavonoids. Among naturally occurring citrus flavonoids, naringin and hesperidin (3',5,7-trihydroxyflavonone 7-rhamnoglucoside) have been pharmacologically evaluated as potential antioxidant, anticancer (Aboobaker *et al.*, 1994). and anti-inflammatory agents (Emin *et al.*, 1994). Furthermore, hesperidin might well be associated with the prevention of artherosclerosis progression (Monforte *et al.*, 1995; Lee *et al*, 1999). It has been reported that hesperidin has an inhibitory effect on prostaglandin synthesis (Damon *et al.*, 1987). Since the alteration of prostaglandin biosynthesis likely modulates bone metabolism, it is possible that these natural flavonoids change bone metabolism. Chiba *et al.* (2003) have reported that hesperidin inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice.

Taken together, these results suggest that flavonoids, such as hesperidin, are important modulators of bone metabolism; however, the mechanism by which they exert their protective effects on bone is unclear. We undertook the present study to determine the possible cellular mechanism and action of hesperidin which modulate bone metabolism.

Materials and Methods

Reagents

The following were used: Dulbecco's modified Eagle medium (DMEM), α-minimum essential medium (α-MEM), fetal bovine serum (FBS), trypsin-EDTA and other tissue culture reagents (Gibco laboratories, Invitrogen Co. Grand Island, USA); Plastic culture wares (Corning, Corning, NY, USA); BCA protein assay reagent (Pierce, Rockford, IL, USA); Hesperidin and all other reagents were purchased from Sigma chemical company (St. Louis, MO, USA). Recombinant human receptor activator of NF-κB ligand (RANKL) and recombinant human macrophage-colony stimulating factor (M-CSF) were purchased from PeproTech (London, UK). The Osteoprotegerin (OPG) ELISA kit and Osteoclast activity assay substrate (OAAS^{TM}) were purchased from Oscotec Inc. (Cheonan, Korea).

Osteoblastic cell culture

ROS17/2.8 and HOS cell lines were derived from rat and human osteosarcoma, respectively. These osteoblastic cell lines were used for the assessment of cell proliferation, cell viability, and alkaline phosphatase (ALP) assay. Another human osteosarcoma cell line, MG63, was used for the assay of OPG secretion. Each cell line was cultured in DMEM containing 10% FBS and antibiotics. The cells were cultured in a 75 cm^2 culture flask and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When they reached confluence, the cells were subcultured at a ratio of 1:10 by suspending the cells with trypsin-EDTA solution. Each cell line was replated in 24-well or 96-well culture plates and used for each experiment.

Measurement of osteoblastic cell proliferation

ROS17/2.8 and HOS cells were plated in 24-well plates (2×10^4 cells/well) and cultured for 48 hours with DMEM containing 10% FBS. Media were replaced with fresh media containing various concentrations of hesperidin (10^{-12}~10^{-8} M) and cultured for an additional 48 hours. The cells were then detached by treatment with trypsin-EDTA solution and resuspended in FBS-containing media. Cell suspension was mixed with equal volume of 0.4% trypan blue solution in phosphate buffered saline (PBS), and the cells were counted and scored for dye exclusion in a hemocytometer under a light microscope.

Measurement of osteoblastic cell viability

Cell viability was assessed by a colorimetric method based on the reduction of 3-[4,5-dimethyl-thiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) by viable cells. ROS17/2.8 and HOS cells were cultured for 48 hours in 96-well plates (5×10^3 cells/well) with DMEM supplemented with 10% FBS.

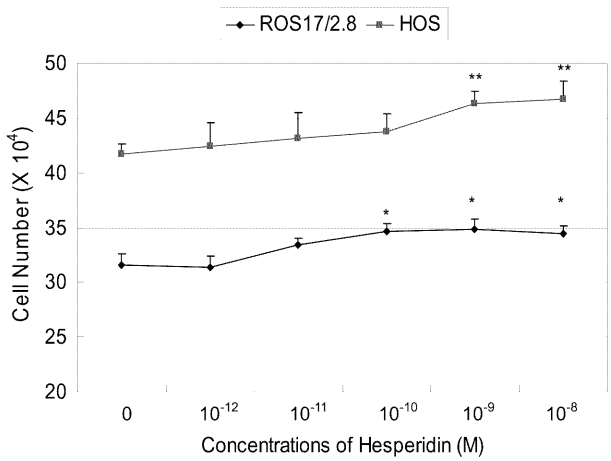

Fig. 1. Effects of hesperidin on the proliferation of osteoblastic cells. Cells were cultured in the presence or absence of hesperidin for 48 hours. After culture, cells were counted for dye exclusion in a hemocytometer under a light microscope. Values are Mean±S.E. (n=8). $^{*}P < 0.05$, $^{**}P < 0.01$.

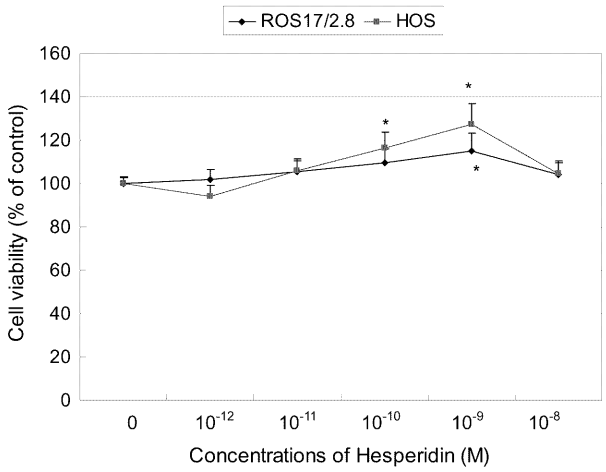

Fig. 2. Effects of hesperidin on the viability of osteoblastic cells. Cells were cultured in the presence or absence of hesperidin for 48 hours. Cell viability was measured at 550 nm as described in materials and methods. Cell viability was expressed with an absorbance ratio. Values are Mean±S.E. (n=10). * $P < 0.05$.

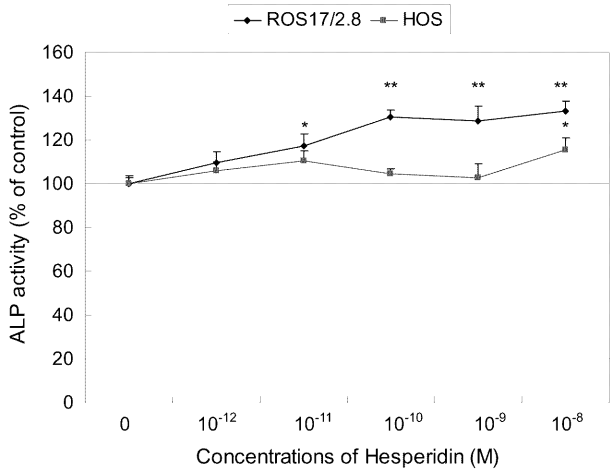

Fig. 3. Effects of hesperidin on the ALP activity of osteoblastic cells. Cells were cultured in the presence or absence of hesperidin for 48 hours. Enzyme activity was measured at 405 nm as described in materials and methods. Enzyme activity was expressed with percent ratio. Values are Mean±S.E. (n=8). * $P < 0.05$, ** $P < 0.01$.

Various concentrations of hesperidin (10^{-12}~10^{-8} M) were added at the start of the culture period. MTT (50 μg/well) was added for the last four hours of culture. After culture, the media were discarded, and the formazan dye formed during the culture was solubilized in isopropanol and quantified in a microplate absorbance reader (SLT 400, SFC, Austria) at 550 nm. Results were expressed as percent viability [100×(absorbance of treated wells/absorbance of control wells)].

Measurement of ALP activity

ALP activity of osteoblastic cells was determined in ROS17/2.8 and HOS cultures after treatment with hesperidin. The osteoblastic cells were plated at a density of 2×10^4 cells/well in 24-well plates and cultured for 48 hours. When the cells reached approximately 70% confluence, they were exposed to various concentrations of hesperidin (10^{-12}~10^{-8} M) for an additional 48 hours. After washing with ice-cold PBS, the cells were lysed with 0.1% Triton X-100 and used for measuring ALP activity. Enzyme activity was measured using p-nitrophenyl phosphate (pNPP, 100 mM) as a substrate. The protein concentration of each sample was measured using a BCA protein assay reagent.

Measurement of OPG secretion

OPG secretion from osteoblasts was determined in MG63 cell cultures after treatment with hesperidin. MG63 cells were plated at a density of 10^4 cells/well in 96-well plates and cultured for 24 hours. Media were replaced with fresh media containing 20 mM $CaCl_2$ (positive control) or with various concentrations of hesperidin (10^{-12}~10^{-8} M) and cultured for an additional 24 hours. After culture, OPG secreted into the culture media was analyzed using a sandwich ELISA kit, according to the method described

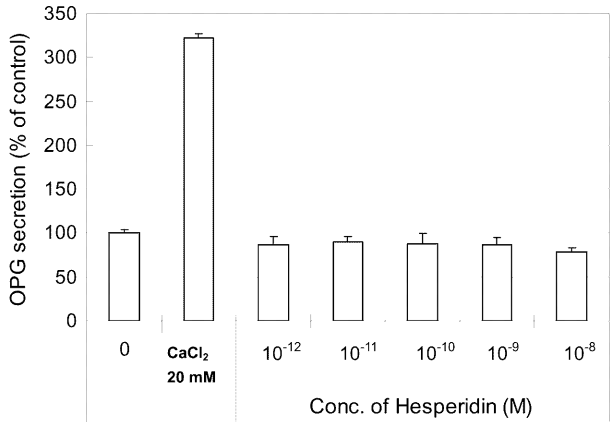

Fig. 4. Effects of hesperidin on the OPG secretion from osteoblastic cells. Cells were cultured in the presence or absence of hesperidin for 24 hours. OPG secretion into the culture medium was measured by the ELISA kit. OPG secretion was expressed with percent ratio. Values are Mean±S.E. (n=10). * $P < 0.05$, ** $P < 0.01$.

previously (Simonet *et al.*, 1997). A standard curve was generated using recombinant human OPG. Briefly, 100 μl of 200-fold diluted medium was incubated in the ELISA plate coated with OPG antibody for two hours. The plate was washed three time with TPBS (0.05% Tween 20 and 1X PBS). Biotinylated anti-OPG antibody was added to each well and incubated for two hours. After washing with TPBS, streptavidin-HRP conjugate was added to each well. Color was developed with a substrate addition, stopped with 1 M phosphoric acid, and analyzed by reading the absorbance at 450 nm.

Mouse bone marrow cell and RAW/264.7 cell culture

The mice were sacrificed by cervical dislocation. The

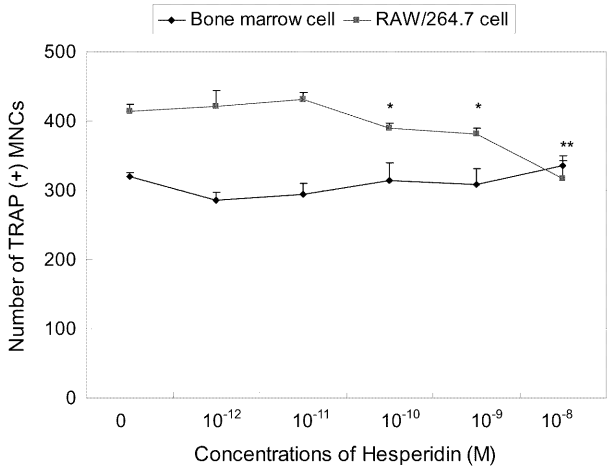

Fig. 5. Effects of hesperidin on the TRAP(+)-MNCs formation in mouse bone marrow cells and RAW/264.7 cell culture. Cells were cultured in 96-well culture plates in the presence of RANKL, M-CSF and hesperidin. After culture, the total TRAP(+)-MNCs were counted from the entire culture surface. Values are Mean±S.E. (n=8). $^{*}P < 0.05$, $^{**}P < 0.01$.

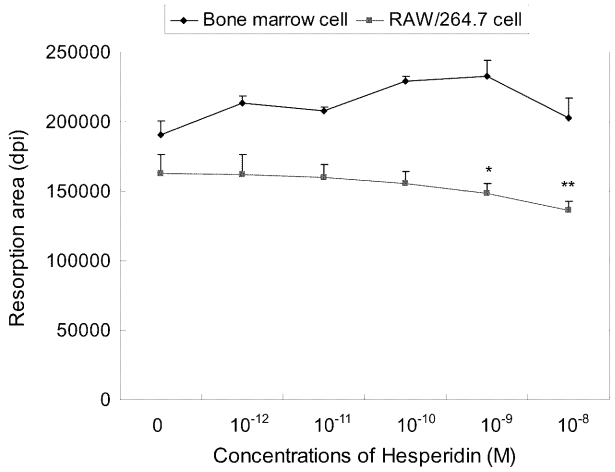

Fig. 6. Effects of hesperidin on the OAAS™ plate resorption in mouse bone marrow cells and RAW/264.7 cell culture. Cells were cultured in 96-well OAAS™ plates in the presence of RANKL, M-CSF and hesperidin. After culture, the total area of the resorption pit was measured. Values are Mean±S.E. (n=8). $^{*}P < 0.05$, $^{**}P < 0.01$.

femur and tibia were removed aseptically, freed of adherent soft tissue, and cut across the epiphysis. The marrow cavity was flushed with 1 ml of enzyme solution containing 0.01% collagenase, 0.05% trypsin, and 0.5 mM EDTA. After washing, the marrow cells were seeded in α-MEM containing 10% FBS and 5 ng/ml M-CSF. After a 24-hour culture, the nonadherent cells were collected and seeded at a density of 5×10^4 cells/well in a 96-well plate or in a 96-well OAAS™ plate for the measurement of osteoclast generation and activity, respectively. RAW/264.7 cells were cultured in DMEM containing 10% FBS and seeded at 5×10^3 cells/well in a 96-well culture plate or a 96-well OAAS™ plate for the measurement of osteoclast generation and activity, respectively.

Measurement of osteoclast generation

After seeding of bone marrow cells or RAW/264.7 cells in a 96-well plate, the cells were cultured in α-MEM, supplemented with 10% FBS, 50 ng/ml RANKL, 10 ng/ml M-CSF, and various concentrations of hesperidin. The cells were cultured for up to seven or eight days while the culture medium was changed on days three and five. After culture, the cells were rinsed with PBS, fixed with citrate-acetone-formaldehyde for five minutes and stained for tartrate-resistant acid phosphatase (TRAP) for the determination of TRAP(+)-multinucleated cells. The cells were stained using a commercial kit (Sigma, 387-A). Total TRAP(+)-multinucleated cells (containing three or more nuclei) were counted from the entire culture surface of the well by light microscope.

Measurement of osteoclast activity

After seeding of bone marrow cells or RAW/264.7 cells in 96-well OAAS™ plates, the cells were cultured in α-MEM, supplemented with 10% FBS, 50 ng/ml RANKL, 10 ng/ml M-CSF, and various concentrations of hesperidin. The cells were cultured for up to seven or eight days while the culture medium was changed on days three and five. The culture medium was removed and the cells were detached to measure the resorption areas by treating the culture plates with 5% sodium hypochlorite for five minutes. The resorption areas were captured using a microscope equipped with a digital camera. The total resorption pit areas of the well surface were measured by image-analyzing software, Image pro plus™ (ver. 3.0, Media Cybernetics, USA).

Statistical analysis

All data represent the mean value obtained from at least eight replicates in each experiment. Data were analyzed using the Student's *t*-test. A P value of less than 0.05 was thought to represent a significant difference between the pairs. The values are presented as mean±S.E.

Results

The number of osteoblastic cells increased when treated with hesperidin. A statistically significant increase in cell numbers was observed with 10^{-10}~10^{-8} M hesperidin treatment in ROS17/2.8 cell culture and 10^{-9} and 10^{-8} M hesperidin treatment in HOS cell culture (Table 1). Cell viability was measured by the MTT reduction assay. Similar to that in the cell proliferation study, the viability of the osteoblastic cells increased when treated with hesperidin. A statistically significant increase in cell viability was observed with 10^{-9} M hesperidin treatment in ROS17/2.8 cell culture and 10^{-10} and 10^{-9} M hesperidin treatment in HOS cell culture (Table 2).

ALP activity, one of the well-known osteoblastic phenotypic markers, was measured after treatment of osteoblastic cells with hesperidin. The ALP activity of osteoblastic cells increased when treated with hesperidin. A statistically significant increase was observed with 10^{-11} - 10^{-8} M hesperidin treatment in ROS17/2.8 cell culture and 10^{-8} M hesperidin treatment in HOS cell culture (Table 3). OPG secretion from MG63 cells was measured because basal secretion levels of OPG from ROS17/2.8 and HOS cells were too low to detect. When the MG63 cells were treated with 20 mM $CaCl_2$ (positive control), OPG secretion increased approximately three-fold. OPG secretion from the MG63 cells decreased when treated with hesperidin, and a statistically significant decrease was observed with 10^{-9} and 10^{-8} M hesperidin treatment (Table 4).

Generation and activity of osteoclasts were observed after culture of the bone marrow cells or RAW/264.7 cell line with RANKL and M-CSF. Numerous osteoclasts were generated when bone marrow cells were cultured in the presence of RANKL (50 ng/ml) and M-CSF (10 ng/ml). Hesperidin treatment had no effect on the osteoclast generation from the bone marrow cells (Table 5). When RAW/264.7 cells were cultured in the presence of RANKL (50 ng/ml), numerous osteoclasts were also generated. In contrast to the bone marrow cells, hesperidin decreased the number of osteoclasts generated from RAW/264.7 cells. A statistically significant decrease was observed with 10^{-10} - 10^{-8} M hesperidin treatment (Table 5). Osteoclast activity was assessed by measurement of the resorption pit area after culture of the bone marrow cells or RAW/264.7 cell line on OAAS™ plate. Osteoclastic activity in the bone marrow cell culture was not affected; however, osteoclastic activity in the RAW/264.7 cell culture decreased with hesperidin treatment. A statistically significant decrease was observed with 10^{-9} and 10^{-8} M hesperidin treatment in the RAW/264.7 cell culture (Table 6).

Discussion

Osteoporosis is a major health concern for aging persons and their communities. The progressive decrease in bone mass leads to an increased susceptibility to fractures, which results in substantial morbidity and mortality (Riggs and Melton, 1992). The ultimate goal of treating osteoporosis is to reduce the risk of fracture by increasing the bone mass to a normal level. Complementary nutritional and pharmacological remedies are needed to prevent bone loss with increasing age. Estrogen replacement therapy, which is effective in preventing postmenopausal bone loss, can be accompanied by numerous side effects (Genant *et al.*, 1989; Recker, 1993). In addition to anti-osteoporotic medicine, a nutritional approach could be an inexpensive means to prevent bone loss after menopause. The chemical compounds in foods that act on bone metabolism, however, have been poorly understood.

In our study, the role of hesperidin, a citrus flavonoid, in bone metabolism was examined by the observation of its effects on the proliferation and activity of osteoblasts and generation, as well as the activity of osteoclasts. The number of ROS17/2.8 and HOS cells increased when treated with hesperidin. A statistically significant increase was observed with 10^{-10} - 10^{-8} M hesperidin treatment in ROS17/2.8 cells and with 10^{-9} and 10^{-8} M hesperidin treatment in HOS cells. Similar to that in the cell proliferation study, the viability of osteoblastic cells increased when treated with hesperidin. ALP is the most widely recognized biochemical marker for osteoblastic activity (Farley and Baylink, 1986). Although its precise mechanism of action is poorly understood, this enzyme is believed to play an important role in bone mineralization (Bellows *et al.*, 1991). In the present study, ALP activity of osteoblastic cells increased when treated with hesperidin. Estrogen influences the growth, differentiation, and functioning of many target tissues (Korach *et al.*, 1994). There have been many reports which have demonstrated that flavonoids such as genistein stimulated ALP activity of osteoblast-like cells, suggesting an anabolic effect on bone formation (Anderson *et al.*, 1998; Fanti *et al.*, 1998; Sugimoto and Yamaguchi, 2000). Phytoestrogens are believed to exert their action by interacting with estrogen receptors; even an alternative mechanism, most notably the inhibition of protein kinase activity, has been proposed (Messina *et al.*, 1994; Kurzer and Xu, 1997). Although it is unclear whether hesperidin can modulate osteoblast proliferation and activity via action on the estrogen receptors, the results obtained in our study suggest that hesperidin could increase the proliferation and activity of osteoblasts. The production of OPG from MG63 cells decreased when treated with hesperidin, and a statistically significant decrease was observed with 10^{-9} and 10^{-8} M hesperidin treatment. OPG is a member of the tumor necrosis factor receptor family that antagonizes the ability of the RANKL (Anderson *et al.*, 1997) to bind to its receptor, RANK (Kong *et al.*, 1999). RANK is a receptor on osteoclasts and preosteoclasts that is essential for their differentiation, activation, and survival. RANKL, a transmembrane molecule, located on bone marrow stromal cells and osteoblasts, binds to RANK, which is located on the surface of osteoclast precursors. This ligand-receptor interaction activates NF-κB, which stimulates differentiation of osteoclast precursors to osteoclasts. OPG, also produced by osteoblasts/stromal cells, binds to RANKL, sequestering and preventing it from binding to RANK, which results in the inhibition of osteoclastogenesis. This study suggests that hesperidin could modulate osteoclastogenesis or bone metabolism by decreasing the OPG production from osteoblasts.

The primary cells responsible for bone resorption are multinucleated osteoclasts, which are present only in bone. In this study, the effects of hesperidin on the generation and activity of osteoclasts in bone marrow cells or RAW/264.7 cell cultures were observed. The generation and activity of

osteoclasts were studied by measuring TRAP(+)-multinucleated cell formation or the area of resorption pit after the culture of osteoclast precursor cells on the OAAS™ plate, respectively. Hsu *et al.* (1999) have shown that RAW/264.7 cells, a mouse myeloid cell line, can be differentiated into osteoclasts with a high yield by stimulation of RANKL. In this study, many TRAP(+)-multinucleated cells were generated when bone marrow cells or RAW/264.7 cells were cultured with RANKL and M-CSF. Resorption pits were also observed when these cells were cultured on the OAAS™ plate. Hesperidin had no effect on the osteoclast generation from bone marrow cells; however, there was a decrease in the osteoclast generation from RAW/264.7 cells. A statistically significant decrease was observed with 10^{-10}-10^{-8} M hesperidin treatment. As with the osteoclast generation study, hesperidin had no effect on the osteoclastic activity in bone marrow cells, but decreased the osteoclastic activity in the RAW/264.7 cell culture, and a statistically significant decrease was observed with 10^{-9} and 10^{-8} M hesperidin treatment. The results obtained in our study with RAW/264.7 cells are in accordance with the study by Chiba *et al.* (2003) who have reported that hesperidin prevented trabecular bone resorption by a decrease in the osteoclast number at the metaphysis of the femur of ovariectomized mice. Our results suggest that hesperidin has a direct inhibitory effect on the osteoclast generation and activity in RAW/264.7 cells, and also has an indirect effect on the osteoclast generation and activity in bone marrow cells by decreasing OPG secretion from mesenchymal cells present in the bone marrow cell culture system. According to our experiment, it seems likely that osteoclastogenesis will be affected by the decrease of OPG secretion from MG63 cells. However, our protocol for culturing RAW/264.7 cells may be an inadequate method to determine the effects caused by changes in OPG secretion levels because cells are directly stimulated with RANKL. Consequently, the RAW/264.7 cell culture system is only sufficient in the determination of direct affects on osteoclasts.

In conclusion, hesperidin increased the proliferation and activity of osteoblasts, while inhibiting the generation and activity of osteoclasts. These cellular effects seem to be helpful for preventing bone loss in many pathologic conditions. Further study is needed for a more complete understanding of the effects of hesperidin on bone metabolism in human beings, particularly in osteoporotic women.

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