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Naringenin inhibits human osteoclastogenesis and osteoclastic bone resorption

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La VD, Tanabe S, Grenier D. Naringenin inhibits human osteoclastogenesis and osteoclastic bone resorption. J Periodont Res 2009; 44: 193–198. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: Naringenin, a naturally occurring flavonoid, possesses a wide range of pharmacological properties. The purpose of this study was to investigate the effect of naringenin on human osteoclastogenesis and osteoclastic bone resorption.

Material and Methods: Naringenin was tested in a human osteoclastogenesis model using primary osteoclast precursor cells activated by receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) for 6 days. Osteoclastogenesis was assessed by determining the number of tartrate-resistant acid phosphatase (TRAP)-stained multinuclear cells, while the secretion of factors involved in osteoclastogenesis was assessed using enzyme-linked immunosorbent assays. The effect of naringenin on bone resorption was investigated using an OsteoAssay human bone plate coupled with an immunoassay to evaluate the release of helical peptide 620–633 from the α1 chain of type I collagen.

Results: Naringenin was non-toxic at the highest concentration used (50 μg/ml). Naringenin (10, 25 and 50 μg/ml) significantly inhibited osteoclastogenesis (by 29 ± 5, 57 ± 8 and 96 ± 1%, respectively). Naringenin also markedly inhibited the secretion of interleukin (IL)-1α (by 59%), IL-23 (by 87%) and monocyte chemoattractant protein-1 (by 58%). Lastly, naringenin (10, 25 and 50 μg/ml) significantly decreased the release of helical peptide 620–633, an indicator of bone resorption activity (by 44 ± 0.5, 73 ± 0.5 and 86 ± 1%, respectively).

Conclusions: Naringenin can inhibit human osteoclastogenesis and osteoclastic bone resorption. It thus holds promise as a therapeutic or preventive agent for bone-related diseases such as periodontitis.

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Bone is continuously remodelled during adulthood through the synthesis of bone matrix by osteoblasts and resorption by osteoclasts (1,2). A balance between bone resorption and bone formation is required for the maintenance of skeletal integrity. Imbalances between osteoblast and osteoclast activities result in skeletal disorders such as osteopetrosis, osteoporosis and inflammatory bone erosion (3,4). Osteoclasts are multinuclear cells generated from haematopoietic monocyte/macrophage precursor cells under the control of two primary cytokines: receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF; 5–7). Perturbations in the expression of inflammatory cytokines, growth factors and hormones can cause an imbalance between osteoblast and osteoclast activities, leading to diseases such as periodontitis (1,2).

Periodontitis is a polymicrobial infection characterized by a destructive inflammatory process that leads to the loss of tooth-supporting tissues, including the periodontal ligament and alveolar bone. While bacteria are the

primary factor in the aetiology of periodontitis, tissue destruction is also a consequence of an uncontrolled host immune response (8–10). Many cytokines secreted during the inflammatory process, including the proinflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), stimulate bone resorption because they contribute to osteoclast differentiation and activation (11).

Natural products play a key role in the field of new drug development. Indeed, several important drugs used in modern medicine come from studies of medicinal plants (12). Bioflavonoids, which are widely distributed in plants, fruits and vegetables, are well known for their health benefits (13). More particularly, naringenin (4′,5,7-trihydroxyflavanone), a naturally occurring flavonoid found in citrus fruits, has a wide range of pharmacological properties including anticancer, antimutagenic and anti-inflammatory activities (14–16). Our laboratory recently showed that naringenin is a potent inhibitor of the pro-inflammatory cytokine response induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) in human macrophages and *ex vivo* whole blood models (17). In the present study, we investigated the effects of naringenin on the differentiation of primary human osteoclast precursor cells activated with RANKL and M-CSF and on osteoclastic bone resorption. In addition, we monitored the secretion of various factors by osteoclast precursor cells involved in osteoclastogenesis.

Material and methods

Material

Naringenin and leukocyte acid phosphatase assay kits were purchased from Sigma Chemical Co. (St Louis, MO, USA). The human Poietics™ osteoclast precursor cell system and OsteoAssay human bone plate were from Lonza Inc. (Allendale, NJ, USA), the Metra™ helical peptide enzyme immunoassay kits were from Quidel Corp. (San Diego, CA, USA), and the MTT [3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay kits were from Roche Diagnostics (Mannheim, Germany).

Treatment of osteoclast precursor cells

Human osteoclast precursor cells derived from haematopoietic stem cells obtained from human bone marrow were activated according the manufacturer's instructions (Lonza Inc.). A complete description, characteristics and cultivation procedures for the osteoclast precursor cells can be obtained at http://www.lonza.com. Briefly, cells were seeded in 96-well plates in triplicate (1×10^3 cells per well in 200 µl) in Osteoclast Precursor Basal Medium (10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, 66 ng/ml of RANKL and 33 ng/ml of M-CSF) supplemented with naringenin (2 µl per well of naringenin prepared in ethanol) at a final concentration of 10, 25 or 50 µg/ml. Cells incubated in the absence of naringenin served as the positive control. Cells were incubated for 6 days (without any change of the culture medium, as recommended by the manufacturer) in a CO_2 incubator in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C to allow complete osteoclast differentiation or for 2 days to assess the cytotoxicity of naringenin.

Cytotoxicity

The cytotoxicity of naringenin was evaluated by an MTT assay according to the manufacturer's protocol. In brief, after the stimulation period, the cells were incubated with MTT for 4 h. The insoluble formazan dye was solubilized overnight at 37°C and the absorbance at 550 nm with the wavelength correction set at 650 nm was measured using a microplate reader. Cytotoxicity was quantified as the relative decrease in the absorbance at 550 nm compared with untreated control cells.

Osteoclast formation

Osteoclast formation was evaluated using a leukocyte acid phosphatase assay kit according to the manufacturer's instructions. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (three or more nuclei) was determined by microscopic observation (×100 magnification). Duplicate counts of triplicate wells for each condition were performed, and the means ± SD were calculated.

Cytokine secretion

Culture supernatants from osteoclast precursor cells incubated with RANKL (66 µ/ml) and M-CSF (33 µ/ml) in the absence or presence of naringenin (10 µg/ml) for 6 days were collected, stored at −70°C and shipped frozen to SearchLight Protein Array Service (Pierce Biotechnology, Woburn, MA, USA) to determine the concentrations of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-17, IL-23, monocyte chemoattractant protein-1 (MCP-1), osteoprotegerin (OPG) and TNF-α, using sandwich enzyme-linked immunosorbent assays (ELISA) combined with piezoelectric printing technology. This procedure uses microplate wells prespotted with highly specific capture antibodies. After incubation, unbound proteins are removed by rinsing, and biotinylated detection antibodies are added. Excess detection antibody is then removed by rinsing prior to adding streptavidin–horseradish peroxidase and chemiluminescent substrate. The enzyme–substrate reaction produces a luminescent signal that is detected with the SearchLight Plus CCD Imaging System. The signal level in the sample spots is proportional to the amount of each specific protein in the standard and is measured by imaging the plate with a cooled CCD camera. Protein concentrations in samples are quantified by comparing the intensity of the spots with the corresponding standard curve using SearchLight Array Amalyst software (Pierce Bio-Technology). Concentrations of proteins in the samples were measured in duplicate at three dilutions, and the mean values of the best measurements were calculated.

Bone resorption

Osteoclasts were generated from primary human osteoclast precursor cells, as described above, using OsteoAssay human bone plates following 6 days of incubation in the absence of naringenin. Thereafter, the culture medium was replaced by fresh medium containing naringenin (2 µl per well of naringenin prepared in ethanol) at a final concentration of 10, 25 or 50 µg/ml. Fresh medium without naringenin was used as a positive control. The cells were incubated for an additional 2 days. The supernatants were collected and stored at −70°C until used. The supernatants were analysed for the presence of helical peptide 620–633 released from the α1 chain of type I collagen, the major substrate of OsteoAssay human bone plate, using a Metra helical peptide enzyme immunoassay kit. The effect of naringenin on bone resorption mediated by activated osteoclasts was recorded as a decrease in the release of collagen peptide compared with untreated control cells.

Statistical analyses

The statistical analyses were performed using paired Student's *t*-test with the level of significance set at $p < 0.05$. The data are presented as means ± SD of triplicate samples.

Results

Cytotoxic effect of naringenin

No significant cytotoxic effects were observed when human osteoclast precursor cells were incubated with naringenin for 2 days. Cell viability was reduced by 10 ± 2% at the highest concentration tested (50 µg/ml).

Osteoclast formation and cytokine secretion

Naringenin inhibited the RANKL/M-CSF-induced differentiation of primary human osteoclast precursor cells into osteoclasts in a dose-dependent manner (Fig. 1). In the absence of naringenin, an almost complete differentiation of precursor cells into typical osteoclasts (three nuclei or more) was observed. The inhibition of osteoclastogenesis by naringenin was not cytotoxicity related, since non-toxic concentrations were used. Naringenin at a final concentration of 10 µg/ml significantly reduced the number of osteoclasts formed (TRAP-positive multinucleated cells; $p < 0.001$ vs. untreated control cells), while osteoclast formation was almost completely inhibited at a final concentration of 50 µg/ml (Fig. 1). More specifically, naringenin at final concentrations of 10, 25 and 50 µg/ml caused an inhibition of 29 ± 5, 57 ± 8 and 96 ± 1%, respectively (Fig. 2).

In order to identify the possible mechanism of inhibition of osteoclast differentiation, we investigated the effect of naringenin on the secretion of mediators involved in osteoclastogenesis. Naringenin (10 µg/ml) markedly decreased IL-1α, IL-23 and MCP-1

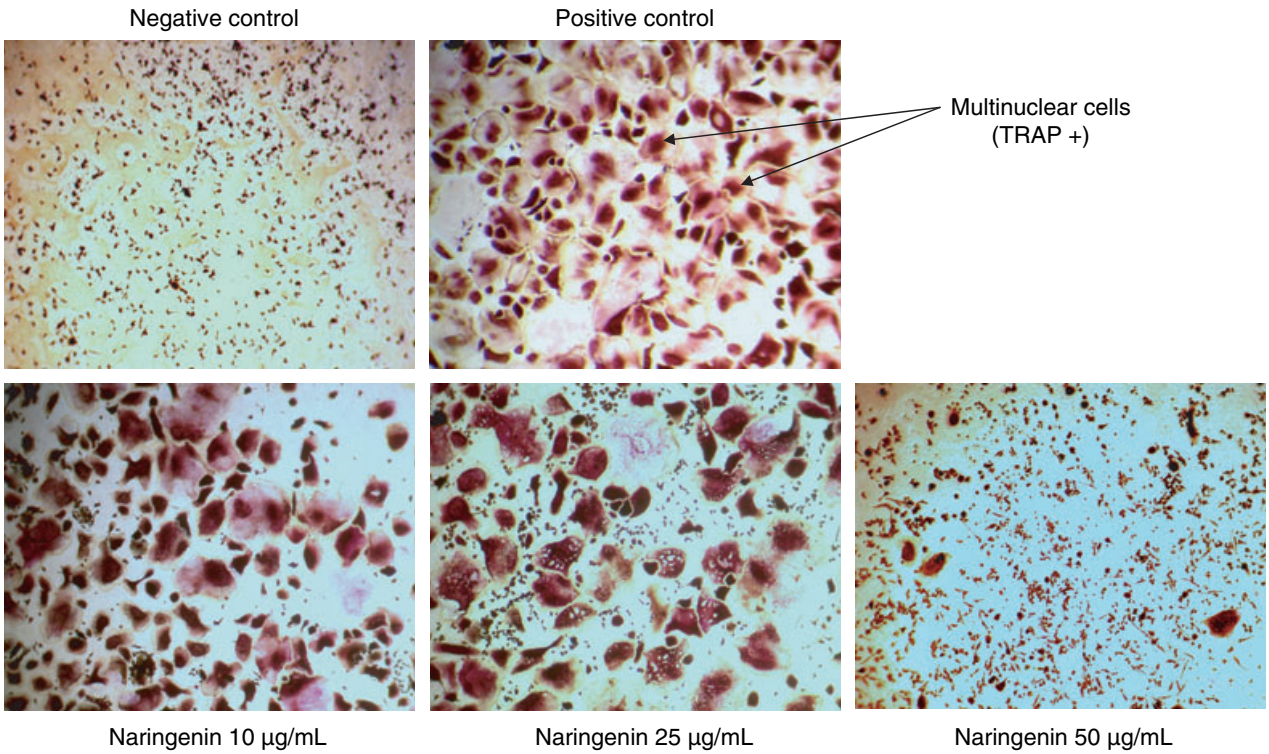

Fig. 1. Effect of naringenin on the differentiation of primary human osteoclast precursor cells into mature osteoclasts. Osteoclast precursor cells were cultured with various concentrations of naringenin in the presence of both RANKL and M-CSF, as described in the Material and methods section. After 6 days, the cells were fixed and stained to detect TRAP-positive multinuclear cells (three or more nuclei). Positive control: presence of both RANKL and M-CSF but no naringenin. Negative control: absence of RANKL. Bar = 500 µm (magnification 100×).

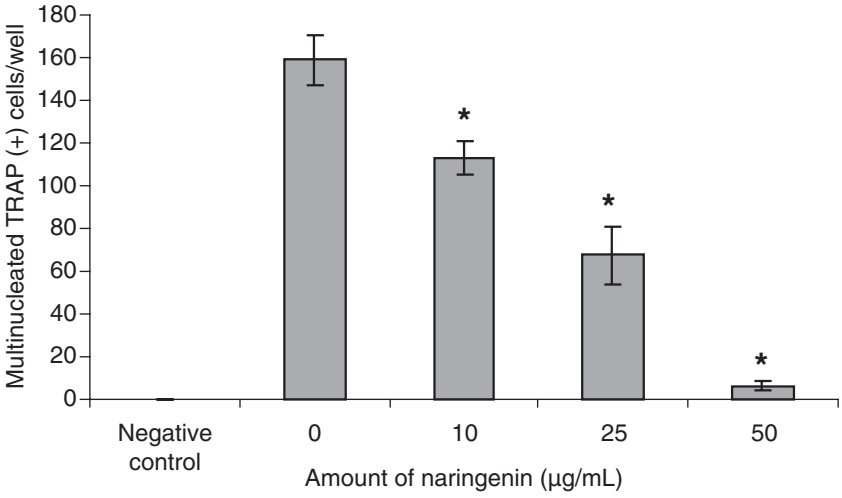

Fig. 2. Effect of naringenin on the differentiation of primary human osteoclast precursor cells into mature osteoclasts induced by RANKL and M-CSF. TRAP-positive multinuclear cells (three or more nuclei) were counted, as described in the Material and methods section. *$p < 0.001$ vs. untreated control cells.

secretion (by 59, 87 and 58%, respectively), while it increased TNF-α and IL-8 secretion (by 58 and 45%, respectively; Table 1). The levels of IL-6 secreted by treated cells and untreated control cells were comparable (Table 1). Lastly, the human precursor osteoclast cells did not release significant amounts of IL-1β, IL-17 or OPG (data not shown).

Osteoclastic bone resorption

The release of helical peptides by osteoclasts was significantly lower in the presence of naringenin at a final concentration of 10 μg/ml ($p < 0.005$) following two additional days of cultivation in the OsteoAssay human bone plate. More specifically, naringenin at final concentrations of 10, 25 and 50 μg/ml caused an inhibition of 44 ± 0.5, 73 ± 0.5 and 86 ± 1%, respectively (Fig. 3).

Discussion

Alveolar bone resorption is a clinical feature of periodontitis. Osteoclasts, which originate from monocyte/macrophage lineage precursors, are actively involved in this destructive process (18). Consequently, regulating osteoclast formation and activity may be one way to prevent and control the progression of periodontitis. Plant extracts and plant-derived molecules have received considerable attention in recent years for their potential health benefits, notably in preventing and inhibiting bone resorption. The flavonoids corylin and bavachin have been reported to stimulate osteoblast proliferation and bone formation and may help prevent osteoporosis (19). Rutin, a quercetin glycoside, preserves bone mass in ovariectomized rats by slowing down *in vivo* bone resorption (20). Quercetin, one of the most commonly occurring flavonoids, reduces osteoclastogenesis in a dose-dependent manner in both murine and human models via a mechanism involving nuclear factor-κB (NF-κB) and activator protein-1 (AP-1; 21).

We recently demonstrated that naringenin, a naturally occurring flavonoid found in citrus fruits, can inhibit the release of pro-inflammatory cytokines by *A. actinomycetemcomitans* LPS in human macrophages and *ex vivo* whole blood models (17). These inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) contribute to osteoclastogenesis by stimulating osteoblasts, synovial fibroblasts and activated T cells to produce RANKL, which in turn induces osteoclast formation and bone resorption (22). We activated human osteoclast precursor cells with RANKL and M-CSF in the presence and absence of naringenin to determine whether naringenin had an effect on osteoclast formation and bone resorption. Our data showed that naringenin inhibited both osteoclast differentiation and bone resorption in a dose-dependent manner at non-cytotoxic concentrations.

The model used in the present study, which uses RANKL- and M-CSF-activated human osteoclast precursor cells, is particularly relevant for studying periodontitis. These conditions may occur *in vivo* in periodontitis patients, who have raised concentrations of RANKL in the gingival crevicular fluid (23,24) and periodontal tissues (25–27) compared with healthy individuals. Furthermore, human periodontal ligament cells are capable of secreting M-CSF and expressing RANKL in response to stimulation with TNF-α (28).

Table 1. Effect of naringenin on cytokine secretion by human osteoclast precursor cells

	Cytokine secretion (pg/ml)*		
Mediators	No naringenin	Naringenin at 10 μg/ml	Percentage change
IL-1α	17.4	7.2	−59
IL-6	15.4	14.8	−4
IL-8	2020	2930	+45
IL-23	279.8	36.6	−87
MCP-1	1955	825	−58
TNF-α	19	30	+58

Cytokines secreted in the conditioned media after 6 days of incubation were quantified by sandwich ELISA microarray.
*Concentrations of proteins in the samples were measured in duplicate at three dilutions and the mean values of the best measurements were calculated.

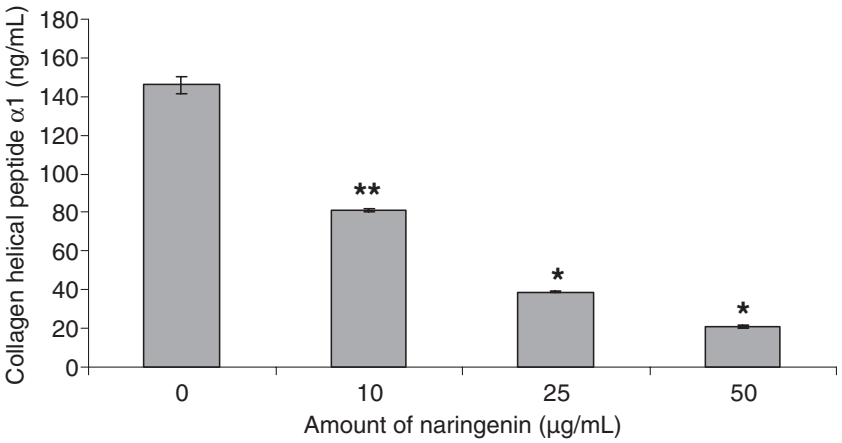

Fig. 3. Effect of naringenin on collagen helical peptide release by osteoclasts formed in an OsteoAssay human bone plate. Collagen helical peptide 620–633 was quantified by ELISA. $*p < 0.001$, $**p < 0.005$ vs. untreated control cells.

To understand the mechanism by which naringenin inhibits RANKL and M-CSF activities, we quantified various factors involved in osteoclastogenesis in the culture supernatants of cells grown in the presence of naringenin and observed a decrease in IL-1α, IL-23 and, MCP-1 secretion. Indeed, these factors were found to contribute to the formation and activity of osteoclasts in human and animal *in vitro* models. Interleukin-1α stimulates the formation of osteoclast-like cells via increased M-CSF and prostaglandin E_2 secretion and decreased OPG production in rat osteoblasts (29), Interleukin-23 stimulates the differentiation of human osteoclasts from peripheral mononuclear cells (30), and MCP-1 increases the number of TRAP-positive multinuclear bone-resorbing osteoclasts in the presence of RANKL from human peripheral blood mononuclear cells (31). The reduction in IL-23 and MCP-1 secretion is likely to be more important in the phenomenon observed, considering the rather low levels of IL-1α produced. An increased secretion of IL-8 and TNF-α was observed in the presence of naringenin. Despite the fact that these two cytokines have been shown to stimulate osteoclastogenesis (11,32), they appear not to play a key role in the differentiation of the osteoclast precursor cells used. Additional studies are required to identify the exact mechanism by which naringenin exerts its beneficial properties.

In conclusion, the present study showed that naringenin inhibits both *in vitro* osteoclast differentiation and bone resorption in a dose-dependent manner. Naringenin thus holds promise as a preventive or therapeutic agent for bone-related diseases such as periodontitis. Interestingly, Wood recently showed that naringenin-supplemented nutrients enhance bone formation during alveolar bone development in young male rats (33). In addition, an herbal-bioflavonoid supplement was reported to prevent periodontal bone loss in a rat model infected with *Porphyromonas gingivalis* and *Actinomyces viscosus* (34).

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