available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Attenuation of osteoclastogenesis and osteoclast function by apigenin

Sanghamitra Bandyopadhyay [a], *Jean-Marc Lion* [b], *Romuald Mentaverri* [b], *Dennis A. Ricupero* [a], *Said Kamel* [b], *Jose R. Romero* [c], *Naibedya Chattopadhyay* [c,*]

[a] *Genetics and Aging Research Unit, Psychiatry Department, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA*

[b] *Unité d'étude des mécanismes de la résorption osseuse et INSERM, ERI-12, Faculté de Pharmacie, 1 Rue des Louvels, 80037 Amiens, France*

[c] *Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

ARTICLE INFO

Article history:
Received 23 March 2006
Accepted 17 April 2006

Keywords:
Flavonoids
Bone resorption
Osteoporosis
Rheumatic arthritis
Tumor necrosis factor
Interferon gamma

ABSTRACT

The physiological effects of the flavone, apigenin on bone cells were studied. We first show that apigenin inhibits tumor necrosis factor alpha (TNFα)- and interferon gamma (IFNγ)-induced secretion of several osteoclastogenic cytokines from MC3T3-E1 mouse calvarial osteoblast cell line. Ligands of the TNF receptor family constitute the most potent osteoclastic cytokines. In MC3T3-E1 cells, apigenin dose-dependently (from 5 to 20 μM) inhibits TNFα-induced production of the osteoclastogenic cytokines, IL-6 (interleukin-6), RANTES (regulated upon activation, normal T cell-expressed and -secreted), monocyte chemoattractant protein-1 (MCP-1) and MCP-3. In addition, apigenin inhibits IFNγ-stimulated secretion of monokines, CXCL-9, and -10 in MC3T3-E1 cells. Next, we show that apigenin strongly inhibits differentiation of 3T3-L1 preadipocytes to adipocytes with attendant inhibition of adipocyte differentiation-induced IL-6, MCP-1, and leptin production. Inhibition of adipogenic differentiation by apigenin could be due to induction of osteogensis as it robustly upregulates mRNA levels of bone morphogenetic protein-6 (BMP-6). Finally, the presence of apigenin inhibited osteoclast differentiation from the RAW 264.7 cell line by reducing receptor activator of nuclear factor kappa ligand (RANKL)-induced expression of tartrate-resistant acid phosphatase (TRAP), RANK, and calcitonin receptor but not CCR1, resulting in the inhibition of multinucleated osteoclast formation. Similarly, apigenin inhibited expression of the osteoclast differentiation markers TRAP, RANK, and c-Fms in osteoclast precursor cells obtained from mouse bone marrow following treatment with RANKL and macrophage colony stimulating factor (MCSF). Furthermore, apigenin induced apoptosis of mature osteoclasts obtained from rabbit long bone and inhibited bone resorption. In all instances, a structurally related compound, flavone had no significant effect. These data suggest that apigenin has multiple effects on all three bone cells that could prevent bone loss in vivo.

* *Corresponding author at:* Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA. Tel.: +1 16177324093.
E-mail address: Naibedya@rics.bwh.harvard.edu (N. Chattopadhyay).
0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.04.018

1. Introduction

Spontaneous increases in the expression and secretion of osteoclastogenic cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα) are associated with estrogen deficiency following menopause [1–4]. Available data now suggest that rheumatoid arthritis (RA), which is a chronic inflammatory disease responsible for local and systemic bone loss, closely resembles post-menopausal osteoporosis, involving the same cascades of osteoclastogenic cytokines (for review, [5]). In the bone marrow, osteoblasts and adipocytes are derived from common mesenchymal stem cells or stromal cells (for review, [6]). Adipocytes can supply the essential stromally derived soluble and cell surface factors necessary for osteoclast differentiation and function in vitro [7]. Recent findings show that high adipocyte count in bone marrow is directly related to bone loss, as fat cells replace osteoblasts [8–11]. Also, adipocyte-produced inflammatory cytokines (adipokines), such as IL-6, MCP-1, and leptin could significantly contribute to bone loss [11,12].

TNFα, produced mainly by activated macrophages, bone marrow stromal cells, and monocytes, acts directly on osteoclasts as well as on various processes leading to osteoclastogenesis [13,14]. Estrogen deficiency results in the expansion of TNFα-secreting T lymphocytes [15,16]. TNF family members have been shown to be the most potent pathophysiologic mediators of ovariectomy-induced bone loss in various mouse models [17–20]. In addition, because TNFα was identified as a pathophysiologic mediator of RA in various mouse models [21,22], anti-TNFα therapy has been used in the treatment of RA. However, since TNFα is a pleiotropic cytokine, anti-TNFα therapy among patients with RA leads to increased risk of mycobacterium infections (especially tuberculosis [23,24]), heart failure, and drug-induced lupus [25].

Osteoblasts play a direct and essential role in overall osteoclast function within the bone microenvironment through the regulation of osteoprotegerin/receptor activators of nuclear kappaB ligand (RANKL)/RANK triad [26,27]. Another mechanism by which TNFα promotes osteoclastogenesis is by activating the osteoblasts to secrete high levels of various osteoclastogenic cytokines such as IL-6 and MCP-1 [28,29]. Furthermore, osteoclast formation, activation, and survival require the interaction of RANK and RANKL, conserved members of the TNF receptor and TNF ligand families, respectively (for review, [30]). Therefore, agents that disrupt TNF receptor family signaling in bone cells specifically are urgently needed.

Interferon gamma (IFNγ) is a member of the T helper (Th) cytokine family and shares Th1 class with TNFα. IFNγ was initially described as an anti-osteoclastogenic cytokine because of its inhibitory role in osteoclastogenesis in vitro [31]. However, it has recently been found to increase bone resorption during estrogen deficiency by promoting T cell proliferation and survival [32]. T lymphocytes significantly contribute to inflammation in RA by secreting IFNγ. In chronic RA patients, IFNγ levels were significantly higher along with TNFα [33]. Recently, CXCL-9/monokine induced by gamma interferon (MIG) was shown to be upregulated by RANKL in osteoclast precursor cells and to promote osteoclast adhesion and migration in an autocrine fashion via its G protein-coupled receptor, CXCR3 [34]. IFNγ-inducible protein-10 (IP-10/CXCL-10) is another ligand of CXCR3. Neutralizing antibodies to IP-10 has been shown to inhibit leukocyte migration and adjuvant-induced arthritis [35]. Therefore, therapeutic attenuation of IFNγ signaling could ameliorate estrogen- and/or RA-induced bone loss.

Flavonoids, diphenyl propanoids found in edible plants, are known to have multiple beneficial biological effects owing to their antioxidant, anti-inflammatory, and estrogenic properties [36–38]. Apigenin (4′,5,7-trihydroxyflavone), a common dietary flavonoid, is a non-toxic and non-mutagenic flavone that can act as a weak estrogen or anti-estrogen by competing with endogenous estrogens for binding to the estrogen receptor [39,40]. Available literature suggests that isoflavonoids such as genistein and daidzein and flavonols such as quercetin significantly reduce bone loss in ovarectomized rats [41,42]. Such effects are likely exerted by inhibiting osteoclastic bone resorption [43–45].

Here we report our assessment of the effects of apigenin in TNFα- and IFNγ-induced production of osteoclastogenic cytokines in osteoblastic MC3T3-E1 cells. We next studied the role of apigenin in adipocyte differentiation from 3T3-L1 cells and production of adipogenic cytokines (adipokines). Finally, we studied the various functions of apigenin in osteoclast differentiation and function. These data suggest that apigenin has a potent anti-osteoclastogenic effect through its action on osteoblasts, preadipocytes, and osteoclasts and raises the possibility of its in vivo use in preventing bone loss from osteoporosis and/or RA.

2. Materials and methods

2.1. Materials

Apigenin and flavone were purchased from Indofine chemicals (Hillsborough, NJ). Apigenin was dissolved in DMSO, aliquoted and stored at −20 °C to avoid oxidation inactivation of the compound. Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA). Recombinant mouse TNFα, rat/murine RANKL, rat/murine macrophage colony stimulating factor (M-CSF), IFNγ, and ELISA kits for murine MCP-1, RANTES, CXCL-9, CXCL-10 and leptin were purchased from R&D Systems (Minneapolis, MN). The ELISA kit for murine MCP-3 was purchased from Bender Medsystems (Burlingame, CA). Mouse cytokine antibody array and IL-6 ELISA kits were purchased from Raybiotech Inc. (Norcross, GA). The adipogenesis assay kit was purchased from Chemicon International Inc. (Temecula, CA), cell proliferation kit I (MTT) and cell death detection ELISA kits from Roche Diagnostic Inc. (Indianapolis, IN), and Micro BCA protein kit from Pierce (Rockford, IL). All other reagents were from Sigma Chemical (St. Louis, MO).

2.2. Culturing MC3T3-E1 and RAW 264.7 cell lines

The mouse calvarial osteoblast cell line, MC3T3-E1, was purchased from American Type Culture Collection (Manassas, VA) and cultured in growth medium containing α-MEM containing 10% heat-inactivated fetal bovine serum (FBS)

and 1% penicillin–streptomycin. The cells were routinely passaged at 80–90% confluency.

The RAW 264.7 monocyte/macrophage mouse cell line was obtained from American Type Culture Collection and was cultured in the growth medium containing α-MEM containing 10% heat-inactivated FBS, 2 mM L-glutamate, and 1% penicillin–streptomycin. For differentiation, RAW 264.7 cells were gently scraped and seeded in either 100-mm^2 plates or 24-well plates at a density of 2.5×10^6 cells/plate or 3×10^3 cells/well (respectively) and cultured for 5 days in growth medium containing 50 ng/ml recombinant murine RANKL. Medium was replaced on the third day, and cells were cultured for 2 more days, at which point large numbers of multinucleated cells were observed. At the end of the culture period, tartrate-resistant acid phosphatase (TRAP)-positive cells with >3 nuclei/cell were counted after staining with a leukocyte acid phosphatase kit (Sigma). For determination of TRAP, RANK, and CTR mRNA levels, RNA was harvested from these cultures.

2.3. Differentiation of 3T3-L1 preadipocyte

3T3-L1 preadipocytes were purchased from ATCC and cultured to confluency in 10% calf serum/DMEM. Two days post-confluency (DAY 0), the cells were treated with the induction media [10% calf serum/DMEM containing 1 μg/ml insulin, 1 μM dexamethasone and 500 μM isobutylmethylxanthine (IBMX)]. Two days after induction medium treatment (DAY 2), the cells were treated with insulin medium [10% calf serum/DMEM containing 1 μg/ml insulin]. Two days later (DAY 4), the medium was changed to 10% FBS/DMEM. The cells were fed with 10% FBS/DMEM every 2 days. Full differentiation was usually achieved by DAY 8. To test the effect of apigenin on the differentiation of 3T3-L1 preadipocytes to adipocytes, 5–20 μM apigenin or flavone (control) were used in parallel culture sets throughout differentiation.

2.4. Adipogenesis assay (Oil Red O spectrophotometric assay)

The kit for adipogenesis assay purchased from Chemicon Inc is based on Oil Red O staining principles, which allows obtaining quantitative information about the inducers and inhibitors of adipogenesis. 3T3-L1 preadipocytes were differentiated in 24-well plates (starting cell number ~60,000/well) in the presence or absence of apigenin or flavone. After the end of 8-day adipogenesis induction (as described above), media were removed and cells were washed twice with PBS. Oil Red O solution (0.5 ml) was added to each well and incubated at room temperature for 15 min. Oil Red O solution was removed, cells were washed three times with 1 ml wash solution, and 0.25 ml dye extraction solution was added to each well. After shaking the plates for 30 min, the dye-extracted solution was transferred to 96-well plates, and absorbance was quantified in a plate reader set at 520 nm.

2.5. MTT cell viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, a colorimetric assay, was used to determine cellular viability following an optimized protocol [46]. After 24 h incubation in reduced serum medium (0.5% FBS) in presence of apigenin or flavone (concentrations indicated in Section 3), cells were incubated with 10 μl MTT (10.4 mg/ml) and incubated at 37 °C in humidified 5% CO_2 for 4 h to convert water-soluble MTT to insoluble formazan, which occurs only in viable cells. After 4 h, 100 μl 50% dimethylformamide with 20% SDS was added to each well to solubilize formazan, and the plates were incubated at 37 °C overnight. Absorbance was then measured at 595 nm with background subtraction at 655 nm.

2.6. In vitro osteoclastogenesis from mouse bone marrow

In vitro osteoclastogenesis was studied using a protocol adapted from that of Koga et al. [47]. Briefly, after sacrifice, mouse bone marrow cells were flushed from long bones using α-MEM supplemented with 10% FBS. After overnight culture in this medium, non-adherent bone marrow cells were seeded in 6-well plates at a density of 250,000 cells/well and cultured for 5–6 days in α-MEM containing 10% FBS, 50 ng/ml RANKL, and 10 ng/ml MCSF. Medium was replaced on the third day. In each well, TRAP-positive cells (>3 nuclei/cell) were then counted after staining with a leukocyte acid phosphatase kit. For determination of TRAP, RANK, and c-Fms mRNA levels, RNA was harvested.

2.7. Primary osteoclasts isolation and culture

Mature osteoclasts were isolated from the long bones of rabbits or mice according to the procedure described elsewhere [48], with slight modifications. Briefly, rabbit long bones were dissected and minced with scissors in α-MEM supplemented with 10% heat-inactivated FBS. The cells were then dissociated from the bone fragments by vigorous vortexing and collected by centrifugation (4 min, 500 rpm) prior to seeding on bovine bone slice or on 24-well plates and incubated overnight in α-MEM supplemented with 10% heat-inactivated FBS. The purified population of osteoclasts used for the apoptotic assay was obtained by removing all of the non-osteoclastic cells from the 24-well plate-seeded bone cells, using a 0.05% solution of collagenase–dispase (20 min, 37 °C). Cell purity was assessed using tartrate-resistant acid phosphatase (TRAP) staining (Leukocyte Acid Phosphatase kit) and was close to 99%. After purification, osteoclasts were left undisturbed for at least 2 h in α-MEM supplemented with 10% FCS and then cultured with test compounds for 48 h in α-MEM supplemented with 1% FCS.

2.8. Pit resorption assay

A previously described protocol optimized in our laboratory was used for the assessment of bone resorbing activity of osteoclasts [44]. Briefly, the unfractionated bone cell preparation was seeded on bovine bone slices. After sedimentation (45 min), medium and non-adherent cells were gently removed and replaced with fresh medium in each well. Remaining cells were incubated for 48 h with various amounts of apigenin or flavone. At the end of the culture period, all cells were removed from the slices and bone resorption was evaluated with double staining using acid hematoxylin solution (1 min) and a solution of 1% toluidine blue–1% borate (30 s). Pit (resorption lacuna) area was then quantified using an

image analysis system (Biocom les Ulis, France) linked to a light microscope (Olympus BH-2, France).

2.9. Osteoclast apoptosis assay

As previously described [44,49], after treatment with the compounds described above, cells were fixed with 3.7% formaldehyde for 5 min and stained with 0.2 mM Hoechst 33258 for 10 min. Cells were examined under a fluorescence microscope for morphological changes of chromatin. At least 100 TRAP-positive multinucleated cells were scored for the incidence of apoptosis. The rate of apoptosis was calculated as the ratio of the number of apoptotic osteoclasts to the total number of counted osteoclasts (apoptotic and non-apoptotic cells).

2.10. Mouse cytokine antibody array

MC3T3-E1 cells were cultured to 80% confluency in a 6-well plate. Cells were serum starved for 4 h, after which cells were incubated in medium (α-MEM containing 0.5% heat-inactivated FBS and 1% penicillin–streptomycin) with 2 ng/ml TNFα or TNFα + 20 μM apigenin for 18 h. The cytokine composition of the conditioned medium was determined using cytokine antibody array following the manufacturer's instruction. The array contained antibodies against CCL21/6Ckine, cutaneous T cell attracting chemokine (CTACK/CCL27), eotaxin, granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony stimulating factor (GM-CSF), IL-2–6, -9, -10, -13, -17, IL-12p40p70, IL-12p70, interferon gamma (IFN-γ), keratinocyte-derived chemokine (KC/mouse homolog of human CXCL-1), leptin, MCP-1/CCL2, MCP-3/CCL7, MCP-5/CCL12, macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), MIP-2, MIP-3β/CCL19, regulated upon activation, normal T cell-expressed and -secreted (RANTES/CCL5), stem cell factor/kit ligand (SCF), soluble TNF receptor I (sTNFRI), thymus- and activation-regulated chemokine (TARC/CCL17), tissue inhibitor of metalloproteinase-1 (TIMP-1), TNFα, thrombopoeitin, and vascular endothelial growth factor (VEGF).

2.11. Measurement of cytokines in cell culture supernatants

MC3T3-E1 cells were seeded in 24-well plates and cultured to 70–80% confluency. The cells were incubated in α-MEM containing 1% penicillin–streptomycin and 0.5% heat-inactivated FBS for 4 h and then incubated with this medium under various conditions (described in Section 3). Supernatants were collected after 18 h and analyzed for IL-6, RANTES, MCP-1, MCP-3, CXCL-9, and CXCL-10 using ELISA kits. To compare various cytokines from MC3T3-E1 cells, each sample was corrected using total protein levels determined by Micro BCA assay.

For measuring IL-6, MCP-1 and leptin from 3T3-L1 cells, cells were plated in 24-well plates (~60,000 cells/well). Eight-day differentiation protocol either in the presence or absence of apigenin/flavone was carried out as described before (Section 2.3). For measuring the above-mentioned cytokines in the conditioned medium of 3T3-L1 cells after differentiation, an 18 h incubation with apigenin and flavone was performed in complete growth medium (10% FBS) at the end of 8-day differentiation protocol. Supernatants were collected and analyzed for IL-6, MCP-1 and leptin. To compare various cytokines from 3T3-L1 cells, each sample was corrected using total protein levels determined by Micro BCA assay.

Table 1 – QPCR primers

Gene name	Accession number	Primers
TRAP	NM_007388	CACCCTGAGATTTGTGGCTGT (F) CGGTTCTGGCGATCTCTTTG (R)
RANK	NM_009399	GCCCAGTCTCATCGTTCTGC (F) TAGCTGTCAGCGCTTTCCCT (R)
CTR	NM_007588	TCAGGAACCACGGAATCCTC (F) ACATTCAAGCGGATGCGTCT (R)
CCR1	NM_009912	CCCAGTGAGAAGAAGGTCAAAGC (F) TGAATCAGAAGCCACAGAGAGC (R)
C-fms	X06368	GCAGGCAGGTCTTACGCAAA (F) CTTAAGCCAGATGCCAGTGGA (R)
BMP-6	NM_007556	CGGTTCTTCAGACTACAACGGC (F) CAATGATCCAGTCCTGCCATC (R)
Cyclophilin	NM_008907	CGAGCTCTGAGCACTGGAGA (F) TGGCGTGTAAAGTCACCACC (R)

2.12. Quantitative real-time PCR

Total RNA was extracted from the cultured cells using Trizol (Invitrogen). cDNA was synthesized from 2 μg total RNA with the Omniscript reverse transcription kit (QIAGEN, Valencia, CA). SYBR green chemistry was used to perform quantitative determinations of the mRNAs for TRAP, RANK, CTR, and a housekeeping gene, cyclophilin A, following an optimized protocol [45,50] using an ABI PRISM 7000 sequence detection system (PE Applied Biosystems). Table 1 shows the primer sequences used. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using Primer Express software (version 2.0.0, Applied Biosystems). The temperature profile of the reaction was 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s, and annealing and extension at 60 °C for 1 min. The size of the PCR product was first verified on a 2.0% agarose gel and then subjected to melting-curve analysis.

2.13. Statistics

Data are presented as means ± S.E. of the indicated number of experiments. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test or Student's *t*-test when appropriate. A *P*-value of <0.05 or less was taken to indicate a statistically significant difference.

3. Results

3.1. Effects of apigenin on TNFα-induced secretion of osteoclastogenic cytokines from MC3T3-E1 osteoblasts

The effect of apigenin and flavone on cell viability was investigated in MC3T3-E1 cells. As a control, the cells were

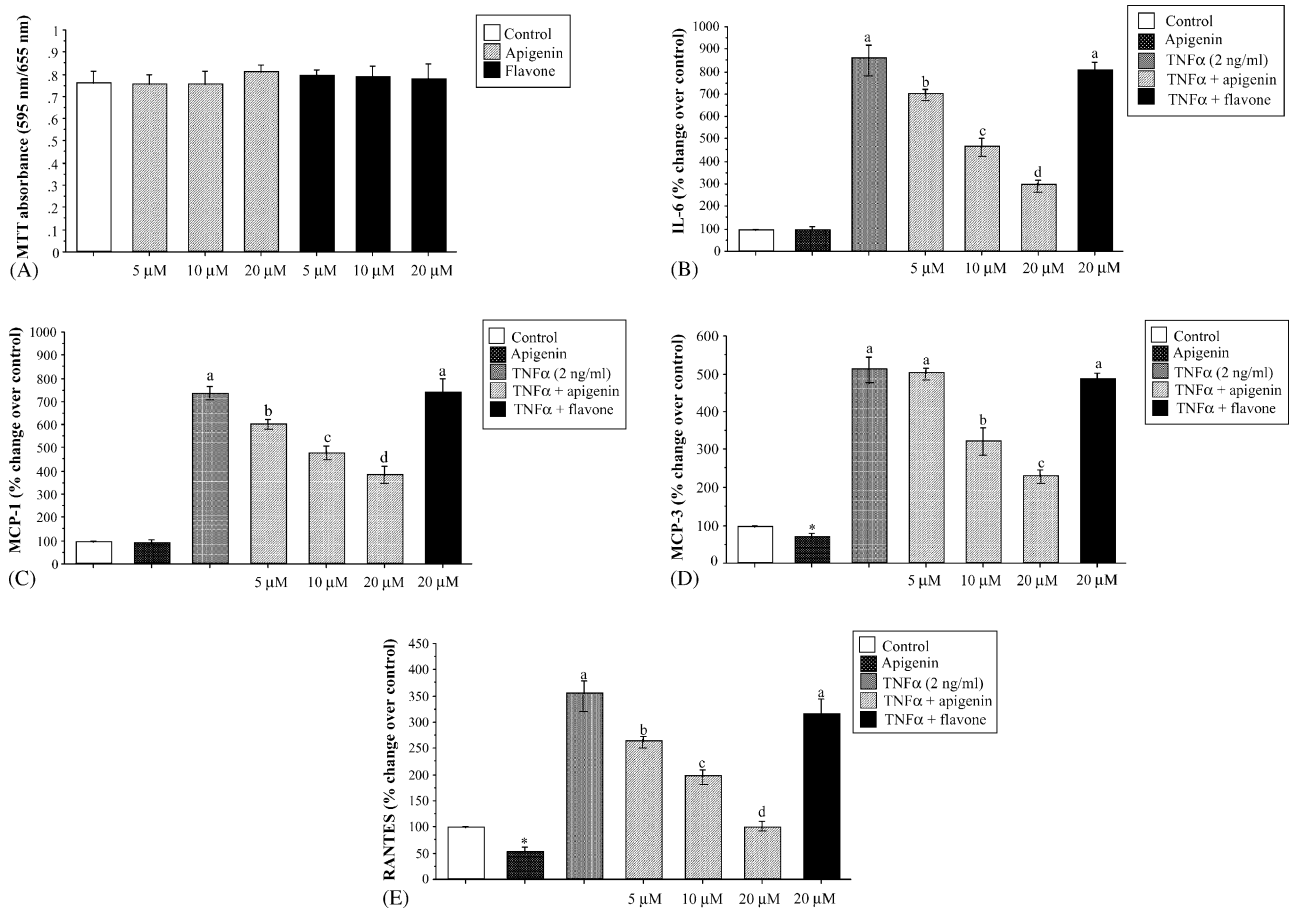

Fig. 1 – Attenuation of TNFα-stimulated secretion of osteoclastogenic cytokines by apigenin in MC3T3-E1 osteoblasts. (A) Effect of incubation with elevated levels of apigenin, on the MTT assay for MC3T3-E1 cells. The cells were treated for 24 h with apigenin and the MTT assay was performed as described in Section 2. There were no differences in the results of the MTT assay for the wells containing cells treated with any of the various concentrations of apigenin and flavone. Amounts of IL-6 [$a > b > c > d >$ vehicle control; $P < 0.05$] (B), MCP-1 [$a > b > c > d >$ vehicle control; $P < 0.05$] (C), MCP-3 [$a > b > c >$ vehicle control and *<vehicle control; $P < 0.05$] (D), and RANTES [$a > b > c > d >$ vehicle control and *<vehicle control; $P < 0.05$] (E) secreted in the conditioned medium of MC3T3-E1 cells after 18 h incubation under various treatment conditions. The data are presented as mean ± S.E. of three experiments each conducted in triplicate.

exposed to vehicle alone (0.1% DMSO). Treatment of MC3T3-E1 cells with 5–20 μM apigenin or flavone for 24 h at reduced serum (0.5% FBS) did not significantly affect the cell viability (Fig. 1A). Therefore, results of the effects of apigenin on various cytokine secretion from these cells presented subsequently (Fig. 1B–E) are not due to reduced cell viability.

TNFα is known to induce synthesis of various osteoclastogenic cytokines from osteoblasts. We first employed a cytokine antibody array to screen for the changes in TNFα-stimulated cytokines in apigenin-treated MC3T3-E1 cells, an established mouse calvarial cell line. Using a mouse cytokine antibody array (containing 32 cytokine antibodies, described in Section 2), we found that 20 μM apigenin reduced TNFα (2ng/ml)-induced secretion of IL-6, MCP-1, MCP-3, and RANTES from MC3T3-E1 cells (data not shown). We confirmed this observation by ELISA for these cytokines. Experiments for Fig. 1B–E were performed in reduced serum condition (0.5% FBS) as described in Section 2.11 for which no significant alterations in cell viability by apigenin was observed (Fig. 1A).

Fig. 1B shows that 20 μM apigenin alone had no effect on basal IL-6 secretion but strongly inhibited TNFα-stimulated secretion in MC3T3-E1 cells. TNFα (2 ng/ml) increased IL-6 secretion by 792 ± 43% ($P < 0.01$), and apigenin dose-dependently inhibited this stimulated secretion by 183 ± 17% ($P < 0.05$), 426 ± 33% ($P < 0.05$), and 628 ± 15% ($P < 0.05$) at 5, 10, and 20 μM, respectively. Flavone (20 μM) had no significant effect on TNFα-stimulated IL-6 secretion by MC3T3-E1 cells.

Fig. 1C shows that 20 μM apigenin alone had no effect on basal MCP-1 secretion but strongly inhibited TNFα-stimulated secretion in MC3T3-E1 cells. TNFα (2 ng/ml) stimulated MCP-1 secretion by 680 ± 21% ($P < 0.01$), and apigenin dose-dependently inhibited this stimulated secretion by 105 ± 23% ($P < 0.05$), 248 ± 27% ($P < 0.05$), and 317 ± 27% ($P < 0.05$) at 5, 10, and 20 μM, respectively. Flavone (20 μM) had no significant effect on TNFα-stimulated IL-6 secretion by MC3T3-E1 cells.

Fig. 1D shows that 20 μM apigenin modestly inhibited basal MCP-3 secretion in MC3T3-E1 cells by 26 ± 8% but had stronger effects in attenuating TNFα-stimulated secretion of MCP-3.

TNFα (2 ng/ml) increased MCP-3 secretion by 455 ± 39% ($P < 0.01$), and apigenin inhibited this stimulated secretion by 183 ± 38% ($P < 0.05$), and 268 ± 14% ($P < 0.05$) at 10 and 20 μM, respectively. Apigenin (5 μM) had no effect on TNFα-stimulated MCP-3 secretion. Flavone (20 μM) had no significant effect on TNFα-stimulated IL-6 secretion by MC3T3-E1 cells.

Fig. 1E shows that 20 μM apigenin in MC3T3-E1 cells inhibited basal RANTES secretion by 43 ± 6% but completely abolished TNFα-stimulated secretion. TNFα (2 ng/ml) increased RANTES secretion by 272 ± 18% ($P < 0.01$), and apigenin inhibited this stimulated secretion by 107 ± 4% ($P < 0.05$), 168 ± 13% ($P < 0.05$), and 268 ± 11% ($P < 0.05$) at 5, 10, and 20 μM, respectively. Flavone (20 μM) had no significant effect on TNFα-stimulated IL-6 secretion by MC3T3-E1 cells.

3.2. *Effects of apigenin on IFNγ-induced secretion of osteoclastogenic monokines from MC3T3-E1 osteoblasts*

We studied the effects of apigenin on IFNγ-induced secretion of osteoclastic monokines CXCL-9 (MIG) and CXCL-10 (IP-10) from osteoblasts. As described in Section 2, 70–80% confluent MC3T3-E1 cells were treated with either 5 ng/ml IFNγ or IFNγ with various concentrations of apigenin, as shown in Fig. 2. Apigenin (20 μM) had no effect on the basal secretion of CXCL-9 from MC3T3-E1 (which was very low), but 5 ng/ml IFNγ increased MIG secretion by 243 ± 41% ($P < 0.05$) (Fig. 2A). Apigenin at 10 and 20 μM inhibited IFNγ-stimulated CXCL-9 secretion by 131 ± 37% ($P < 0.05$) and 241 ± 9% ($P < 0.05$), respectively (Fig. 2A). Flavone (20 μM) had no significant effect on IFNγ-stimulated CXCL-9 secretion from MC3T3-E1 cells.

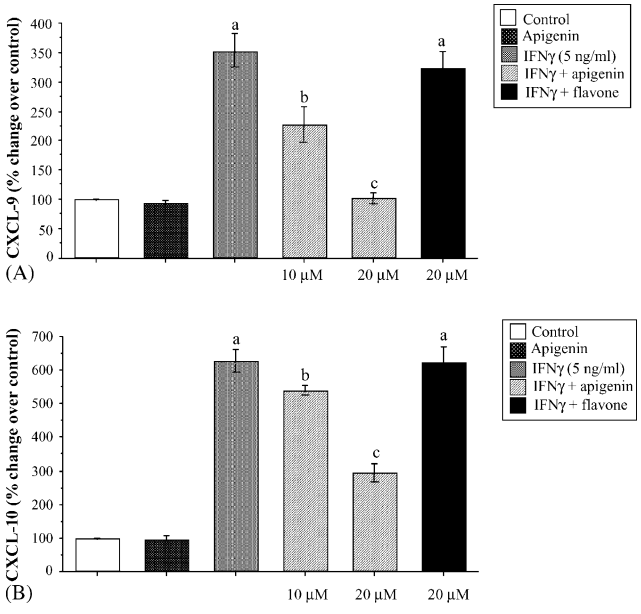

Fig. 2 – Attenuation of IFNγ-stimulated secretion of monokines, CXCL-9 and -10 by apigenin in MC3T3-E1 osteoblasts. Amounts of CXCL-9 [$a > b > c >$ vehicle control; $P < 0.05$] (A) and CXCL-10 [$a > b > c >$ vehicle control; $P < 0.05$] (B) secreted in the conditioned medium of MC3T3-E1 cells after 18 h incubation under various treatment conditions. The data are presented as mean ± S.E. of three experiments each conducted in triplicate.

Next we observed that, whereas 20 μM apigenin had no effect on the basal secretion of CXCL-10 from MC3T3-E1, 5 ng/ml IFNγ robustly increased CXCL-10 secretion by 542 ± 32%, ($P < 0.05$) (Fig. 2B). Apigenin at 10 and 20 μM inhibited IFNγ-stimulated IP-10 secretion by 104 ± 15% ($P < 0.05$) and 308 ± 22% ($P < 0.05$), respectively. Again, 20 μM flavone had no significant effect on IFNγ-stimulated CXCL-10 secretion from MC3T3-E1 cells.

3.3. *Effects of apigenin on the differentiation of 3T3-L1 preadipocytes*

3T3-L1 preadipocytes were differentiated to adipocytes using insulin, dexamethasone, and IBMX as described in Section 2. The differentiation protocol required 8 days to obtain >80% adipocyte differentiation in culture. First the effect of apigenin and flavone on 3T3-L1 cell viability was assessed under adipocyte differentiation conditions. Fig. 3A shows that treatment of 3T3-L1 preadipocytes with 5–20 μM apigenin or flavone in the presence of adipocyte differentiation medium for 8 days did not significantly affect cell viability.

In order to determine whether apigenin has any effect on adipocyte differentiation, cells differentiated in 5–20 μM apigenin was used for the entire period of differentiation and compared with control cells differentiated without apigenin. Flavone (20 μM) was used as negative control. At the end of 8 days incubation, lipid-bound Oil Red O was extracted, and optical density of samples was determined at 490 nm. Fig. 3B shows that apigenin inhibited adipogenesis of 3T3-L1 preadipocytes by 48 ± 6% ($P < 0.05$), 75 ± 4%($P < 0.05$), and 89 ± 3% ($P < 0.05$) at 5, 10, and 20 μM, respectively, compared to control cells (Fig. 3B). Again, 20 μM flavone had no significant effect on adipogenesis (Fig. 3B).

Overexpression of BMP-6 in mesenchymal stem cells has been shown to induce osteoinductive effects [51]. Exogenous BMP-6 stimulates osteogenic differentiation which is independent of estradiol [52]. We next studied whether inhibition of adipogenesis by apigenin in 3T3-L1 cells is accompanied by increase in osteogenic potential. Fig. 3C shows that at a time when apigenin inhibits adipogenesis in 3T3-L1 cells, it stimulated mRNA levels of BMP-6 by 41 ± 5% ($P < 0.05$) and 78 ± 7% ($P < 0.05$) at 5 and 10 μM, respectively, compared to control cells. Flavone (10 μM) had no significant effect on BMP-6 mRNA levels compared with the control (Fig. 3C).

3.4. *Effects of apigenin on the secretion of osteoclastogenic cytokines from 3T3-L1 cells*

Differentiation of preadipocyte to adipocyte is marked by the appearance of cytokines such as leptin, IL-6, and MCP-1. All these cytokines favor various stages of osteoclastic function. We next studied whether apigenin inhibits production of these three cytokines from 3T3-L1 preadipocytes differentiated to adipocytes. The differentiation protocol and apigenin incubation strategy remained exactly similar to the adipogenesis assay described in Fig. 3. On day 8, cells were washed with growth medium and incubated with or without apigenin (5–20 μM) in complete growth medium (see Section 2.11). Flavone (20 μM) served as negative control. Following 20 h incubation, conditioned medium was used for determination of IL-6, MCP-1, and leptin by ELISA.

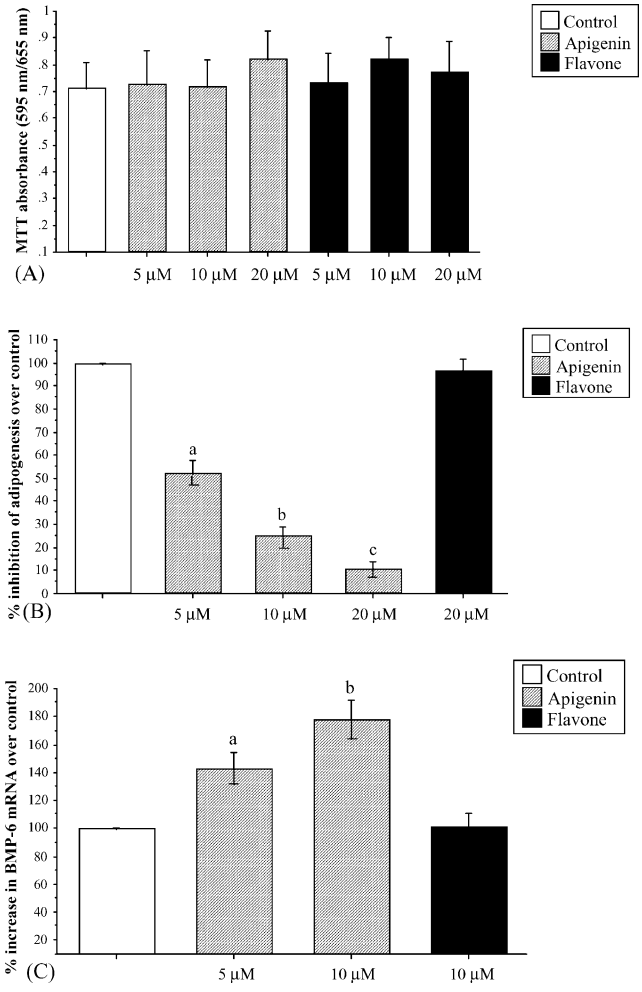

Fig. 3 – Apigenin inhibits adipogenesis of 3T3-L1 cells but upregulates BMP-6 mRNA levels. (A) Effect of incubation with elevated levels of apigenin, on the MTT assay for 3T3-L1 cells. There were no differences in the results of the MTT assay for the wells containing cells treated with any of the various concentrations of apigenin and flavone. (B) Adipogenesis under various treatment conditions was quantitated by extracting lipid-bound Oil Red O and optical density of samples were determined at 490 nm as described in Section 2. Data are expressed as percent inhibition in OD by apigenin treatment over control. $a > b > c >$ vehicle control; $P < 0.05$. (C) QPCR determination of BMP-6 mRNA in 3T3-L1 cells under various treatment conditions was performed as described in Section 2. $b > a >$ vehicle control; $P < 0.05$. The data are presented as mean ± S.E. of three experiments each conducted in triplicate.

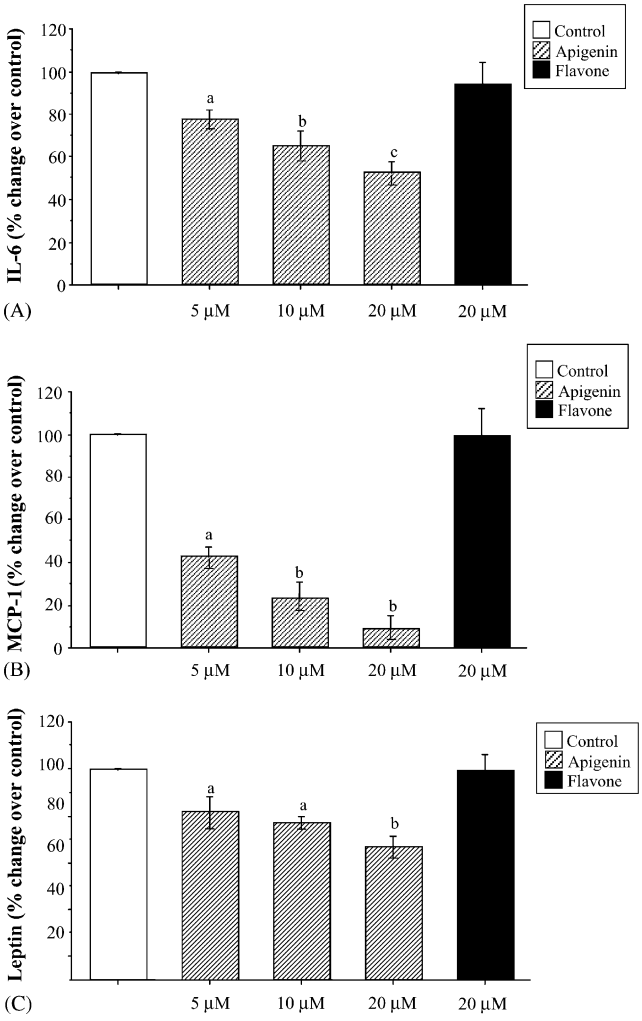

Fig. 4 – Apigenin inhibits secretion of osteoclastogenic cytokines from 3T3-L1 cells. 3T3-L1 cells were treated for induction of adipocyte differentiation (8 days of differentiation) in the presence or absence of apigenin as described in Section 2. Cells were once washed with complete growth medium and further incubated for 20 h under various treatment conditions. Amounts of IL-6 [$a > b > c >$ vehicle control; $P < 0.05$] (A), (B) MCP-1 [$a > b >$ vehicle control; $P < 0.05$] (B), and leptin [$a > b >$ vehicle control; $P < 0.05$] (C) secreted in the conditioned medium of 3T3-L1 cells after 20 h incubation under various treatment conditions. The data are presented as mean ± S.E. of three experiments each conducted in triplicate.

Fig. 4A shows that apigenin inhibited IL-6 secretion by 22 ± 4% ($P < 0.05$), 34 ± 7% ($P < 0.05$), and 47 ± 5% ($P < 0.05$) at 5, 10, and 20 μM, respectively, compared to control cells. Fig. 4B shows that apigenin inhibited MCP-1 secretion by 57 ± 4% ($P < 0.05$), 77 ± 7% ($P < 0.05$), and 93 ± 6% ($P < 0.05$) at 5, 10, and 20 μM, respectively, compared to control cells. Fig. 4C shows that apigenin inhibited leptin secretion by 18 ± 6% ($P < 0.05$), 23 ± 3% ($P < 0.05$), and 31 ± 6% ($P < 0.05$) at 5, 10, and 20 μM, respectively, compared to control cells. Flavone (20 μM) had no significant effect on IL-6, MCP-1 and leptin secretion.

3.5. *Effects of apigenin on differentiation of osteoclasts from their precursor cells*

RANKL, a member of the TNFα family, is secreted by osteoblasts and stimulates formation of multinucleated TRAP-positive osteoclasts. First, RAW 264.7 cells treated with RANKL (50 ng/ml) was tested for cell viability in the presence

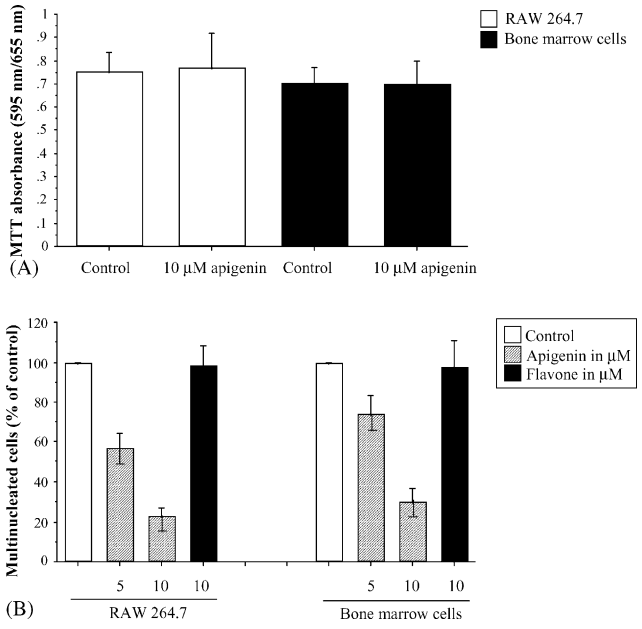

Fig. 5 – Apigenin attenuates formation of multinucleated osteoclasts from mononuclear precursor cells. (A) Effect of incubation with elevated levels of apigenin, on the MTT assay for RAW 264.7 cells treated for 5 days with either RANKL (50 ng/ml) or RANKL + various concentrations of apigenin/flavone. There were no differences in the results of the MTT assay for the wells containing cells treated with any of the various concentrations of apigenin and flavone. (B) Apigenin (5 and 10 μM) attenuates RANKL-induced formation of TRAP-positive multinucleated cells from RAW 264.7 cells at 5 days. Similar concentrations of apigenin attenuate RANKL + MCSF-induced formation of TRAP-positive multinucleated cells (>3 nuclei/cell) from mouse bone marrow cells. *<RANKL-treated cells; $P < 0.05$. The data are presented as mean ± S.E. of three experiments each conducted in triplicate.

of 10 μM apigenin. Fig. 5A shows that 5-day continuous treatment of apigenin did not significantly affect cell viability. Similarly, mouse bone marrow cells treated with RANKL (50 ng/ml) + MCSF (10 ng/ml) for 6 days with continuous presence of 10 μM apigenin did not show any alteration in cell viability (Fig. 5A).

Using RAW 264.7 cells, we next determined the effect of apigenin on RANKL-induced formation of multinucleated cells. Fig. 5B shows that 5- and 10-μM apigenin inhibited RANKL-induced formation of multinucleated cells by 43 ± 7% ($P < 0.01$) and 77 ± 4% ($P < 0.01$), respectively. Flavone (10 μM) had no significant effect on RANKL-induced formation of TRAP-positive cells. Similarly, bone marrow cells treated with 50 ng/ml RANKL + 10 ng/ml MCSF in the presence of 5- and 10-μM apigenin inhibited formation of multinucleated cells by 36 ± 9% ($P < 0.05$) and 70 ± 7% ($P < 0.05$), respectively. Flavone (10 μM) had no significant effect on the formation of TRAP-positive cells from the bone marrow cells treated with RANKL and MCSF.

Increased osteoclastogenesis is accompanied by increased expression of osteoclast differentiation genes such as RANK, TRAP, and calcitonin receptor (CTR). We compared relative expression of these genes in RANKL-induced differentiation of RAW 264.7 cells in the presence or absence of apigenin following 3–5 days' exposure. QPCR determination of TRAP, RANK, CTR, and CCR1 mRNA levels was made and the data expressed as percent increase over undifferentiated RAW 264.7 cells. RANKL-induced expression of TRAP, RANK, and CTR mRNA levels were robustly attenuated by apigenin but not CCR1 (Table 2). Flavone (10 μM) did not significantly alter RANKL-induced expression of any mRNAs studied here (Table 2).

We next studied the effects of apigenin on the induction of TRAP, RANK, and c-fms (also known as colony stimulating factor receptor-1) mRNAs in freshly isolated murine bone marrow cells (see Section 2 for protocol) treated with RANKL and MCSF for 6 days. QPCR determination of TRAP, RANK, and c-fms mRNA levels was made and the data expressed as percent increase over undifferentiated bone marrow cells. At 5

Table 2 – QPCR determination of expression of osteoclastogenic mRNAs

Gene name	RANKL (50 ng/ml)	RANKL + 5 μM apigenin	RANKL + 10 μM apigenin	RANKL + 10 μM flavone
RAW 264.7 cells				
TRAP	574 ± 33*	343 ± 24**	221 ± 14**	556 ± 28*
RANK	477 ± 25*	203 ± 16**	144 ± 18**	462 ± 21*
CTR	257 ± 17*	196 ± 12**	120 ± 22**	262 ± 23*
CCR1	533 ± 22	492 ± 27	540 ± 17	480 ± 38

Gene name	RANKL (50 ng/ml) + MCSF (10 ng/ml)	RANKL + MCSF + 5 μM apigenin	RANKL + MCSF + 10 μM apigenin	RANKL + MCSF + 10 μM flavone
Mouse bone marrow cells				
TRAP	486 ± 19*	291 ± 14**	152 ± 9**	465 ± 20**
RANK	313 ± 8*	201 ± 12**	137 ± 12**	332 ± 34**
c-fms	346 ± 7*	237 ± 8**	132 ± 4**	322 ± 21**

Expressed as percent increase over undifferentiated cells. The data represent the mean ± S.E. (%) of three experiments, each conducted in duplicate.

* $P < 0.01$.

** $P < 0.05$.

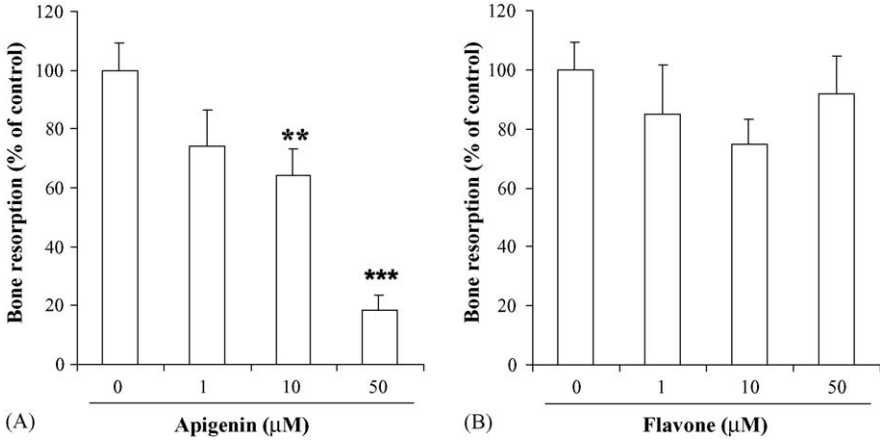

Fig. 6 – Apigenin inhibits resorption mature osteoclasts. Rabbit bone cells were cultured for 48 h in α-MEM in the absence or the presence of various amounts of apigenin or flavone (negative control) from 1 to 50 μM. (A) Bone resorption was assessed by pit area measurement, and results (expressed as percent of control) represent the mean ± S.E.M. of four independent experiments ($n = 11$). * > **; $P < 0.001$ compared to control. (B) Flavone had no effect on osteoclastic resorption.**

and 10 μM concentrations, apigenin robustly inhibited induction of TRAP, RANK, and c-fms mRNAs by RANKL and MCSF (Table 2). None of the RANKL- and MCSF-induced mRNA levels were altered by 10 μM flavone (Table 2).

3.6. *Effects of apigenin on primary osteoclasts*

To investigate the effects of apigenin on bone resorption, osteoclasts were prepared from rabbit long bones. Unfractio-

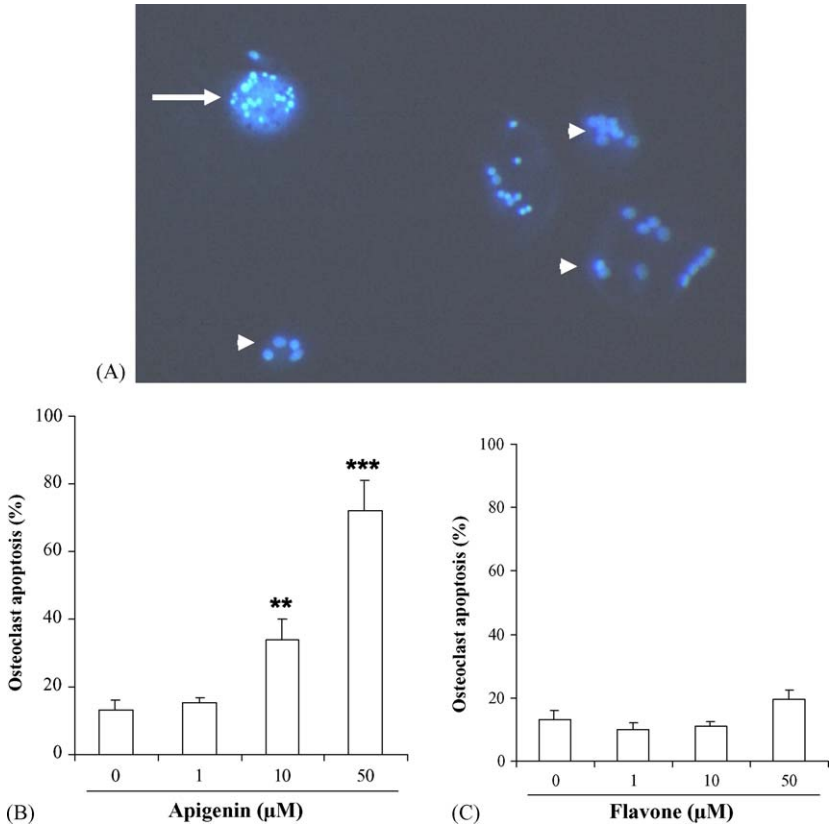

Fig. 7 – Apigenin promotes apoptosis of mature osteoclasts: purified mature osteoclasts were cultured for 48 h in α-MEM in the absence or the presence of various amounts of apigenin or flavone (negative control) from 1 to 50 μM. Apoptosis was detected by staining cells with 0.2 mM Hoechst 33258 to visualize chromatin condensation. (A) A representative fluorescence micrograph (magnification, 400×) showing normal (arrow head) and apoptotic osteoclast (arrow) by 10 μM apigenin treatment. (B) Results are expressed as the mean ± S.E.M. of three independent experiments ($n = 18$). ** > *; $P < 0.01$ compared to controls.**

nated rabbit bone cells were cultured on bone slices for 48 h in presence of several concentrations (1–50 μM) apigenin or flavone (negative control) and bone resorption evaluated by pit area measurement. Fig. 6 shows that whereas apigenin significantly inhibited osteoclastic resorption at 10 and 50 μM, flavone had no effect.

We then studied whether apigenin can modulate osteoclast apoptosis. For this purpose, rabbit osteoclasts were purified from unfractionated bone cells using a method which yielded a very high cell purity in past [44]. Osteoclasts displaying characteristics of apoptosis as chromatin condensation and DNA-fragmentation can be easily distinguished from normal cells by using Hoechst staining and detection with fluorescence microscopy (Fig. 7A). Furthermore, as shown in Fig. 7B, when purified osteoclasts were exposed to increasing concentrations of apigenin (from 1 to 50 μM), osteoclasts underwent apoptosis in a dose-dependent manner whereas flavone had no effect (Fig. 7C).

4. Discussion

We characterized physiological effects of apigenin in osteoblasts, preadipocytes, and osteoclasts, the cells involved in bone remodeling. Our report shows that apigenin modulates function of these bone cells, whose net effect appears to inhibit bone resorption. In osteoblasts, apigenin antagonizes actions of TNFα and IFNγ in inhibiting production of various osteoclastogenic cytokines. In preadipocytes, apigenin inhibits adipocyte differentiation, production of osteoclastogenic cytokines and likely favors osteoblastic differentiation by upregulating BMP-6. In osteoclasts, apigenin inhibits differentiation of preosteoclasts to mature osteoclasts, inhibits bone resorption, and induces apoptosis of mature osteoclasts. Effect of apigenin was concentration-dependent and a structurally related compound, flavone, failed to modify any of the effects of apigenin. These results support apigenin's specificity of action.

In osteoblasts, apigenin potently attenuates TNFα-stimulated secretion of IL-6, MCP-1, MCP-3 and RANTES, cytokines known to promote osteoclastogenesis, without affecting cell viability. The efficiency of attenuating TNFα-stimulated secretion of these cytokines by apigenin was IL-6 > RANTES > MCP-1 > MCP-3. Despite the strong inhibitory action of apigenin, TNFα appears to stimulate all cytokines studied here (albeit at much reduced levels) except for RANTES, in which case apigenin completely abolishes TNFα stimulation. In the cases of IL-6 and MCP-1, apigenin selectively inhibits TNFα-stimulated secretion, whereas for MCP-3 and RANTES, apigenin inhibits both basal and TNFα-stimulated secretions. The basal inhibition of secretion of these two cytokines by apigenin is unlikely to result in their attenuation of TNFα-stimulated secretion, as the extent of inhibition in the later case is much more than the former. Recently, we have shown that kaempferol, a flavonol, inhibits TNFα-stimulated IL-6 and MCP-1 from osteoblasts [45]. From our current study, it appears that apigenin is more effective than kaempferol in antagonizing TNFα action on osteoblasts, as apigenin inhibits TNFα-stimulated RANTES and MCP-3 levels in addition to inhibiting IL-6 and MCP-1 production. The cumulative effects of inhibiting these TNFα-stimulated osteoclastogenic cytokines from osteoblasts by apigenin could provide more efficient attenuation of osteoclastogenesis and bone resorption in vivo than kaempferol.

Apigenin is also classified as a phytoestrogen. However, estrogen itself does not disrupt TNFα-stimulated actions on osteoblasts. For example, in osteoblasts, 17β-estradiol does not inhibit TNFα-induced synthesis of IL-6 [53,54] and fails to modulate Fas-mediated apoptosis induced by TNFα [55]. Our data show that apigenin specifically inhibited TNFα-stimulated IL-6 and MCP-1 secretion from the osteoblasts without altering their basal secretion. Moreover, flavone, a structurally related compound, had no effect in any of the effects observed by apigenin. Thus, apigenin's action on TNFα-stimulated production of various cytokines studied here appears to be independent of the estrogen receptor.

Recently, apigenin and another flavone, luteolin, from 3.5 to 35.0 μM have been shown to inhibit antigen-specific IFNγ production in vitro from autoimmune T cells derived from murine and human sources [56]. Bone loss during osteoporosis and RA is known to occur through the production of IFN and various other factors by activated T lymphocytes. IFNγ stimulates production of CXCL-9 (MIG) and CXCL-10 (IP-10) from osteoblasts, which in turn modulates T lymphocyte proliferation and recruitment by binding with CXCR3 receptor on T lymphocytes (for review, [57]). In the context of RA, these events facilitate bone resorption. Our data show that IFNγ had a more robust stimulatory effect on the secretion of CXCL-10 from MC3T3-E1 cells than CXCL-9. However, apigenin completely abolished IFNγ-stimulated CXCL-9 secretion, whereas IFNγ still stimulated CXCL-10 secretion albeit at much reduced levels. Therefore, at the osteoblast level, our data demonstrate that, by inhibiting IFNγ-stimulated secretion of CXCL-9 and -10 from osteoblasts, apigenin can inhibit osteoclastogenesis.

Adipocytes and osteoblasts share common multipotential mesenchymal stem cells in bone marrow [6]. Aging bone marrow is characterized by a reciprocal decrease of osteogenesis and an increase in adipogenesis [10]. Therefore, inhibiting bone marrow adipogenesis and enhancing osteogenesis are therapeutic goals in the treatment of osteopenic disorders such as osteoporosis [11]. Our data show that apigenin (but not the structurally related flavone) strongly inhibits differentiation of 3T3-L1 preadipocytes into adipocytes. Since 3T3-L1 differentiation to adipocyte is accomplished by treatment with a mixture of insulin, dexamethasone, and IBMX, whether apigenin specifically blocks any one or all of them need to be determined. In addition to inhibiting adipogenesis, our data also reveal that apigenin robustly stimulates mRNA levels of BMP-6 in 3T3-L1 cells. BMP-6 upregulation favors osteogenic differentiation of mesenchymal stem cells [51]. BMP-6 is also known to promote osteogenic differentiation of mesenchymal stem cell by an estradiol-independent mechanism [52]. Therefore, upregulation of BMP-6 by apigenin suggests that inhibition of adipogenesis could be due to stimulation of osteogenesis by apigenin. Additional studies are required to address this possibility.

Marrow adipocytes support osteoclastogenesis. When cocultured with preadipocyte or adipocyte-enriched BMS2 stromal layers, primary bone marrow cells undergo osteoclast differentiation and maturation [7]. One of the mechanisms of

supporting osteoclastogenesis is by the production of a variety of cytokines and chemokines, such as leptin, TNFα, IL-6, MCP-1, and others (for review, [58]). Our data show that, as a likely consequence of inhibiting differentiation, apigenin potently inhibits secretion of leptin, IL-6 and MCP-1 by 3T3-L1 cells. Efficiency of inhibiting secretion of these cytokines by apigenin was MCP-1 > IL-6 > leptin. MCP-1 expression is known to be markedly upregulated in 3T3-L1 cells following induction of adipocyte differentiation [12] and apigenin inhibits this upregulation potently. Leptin has been identified as a strong inhibitor of bone formation (for review, [59]). Therefore, in association with the inhibition of IL-6 and MCP-1 by apigenin, inhibition of leptin secretion is likely to have beneficial effects on bone health.

Recently, we have shown that flavonols such as kaempferol and quercetin inhibit osteoclast differentiation by antagonizing RANKL action in RAW 264.7 cells and promote osteoclast apoptosis [44,45]. Here we demonstrate that apigenin robustly diminished the formation of multinucleated giant cells from RAW 264.7 and bone marrow cells but did not induce cell death. Apigenin strongly inhibited the expression of RANKL-induced differentiation genes such as RANK, TRAP, and CTR in RAW 264.7 cells. The effect of apigenin in inhibiting expression of these three genes does not appear to be due to generalized transcriptional repression as apigenin fails to inhibit the expression of CCR1, the receptor for RANTES, which is highly upregulated in differentiated osteoclasts [50,60].

We extended the results obtained from RAW 264.7 cells to mouse bone marrow cells. Differentiation of bone marrow cells by RANKL and MCSF resulted in a robust increase in the expression of TRAP, RANK, and c-fms mRNAs, but apigenin blocked this induction, suggesting that the action of apigenin observed in the cell line is reproduced in primary culture system. Since apigenin attenuates differentiation of osteoclast precursor cells in vitro, it may be efficacious in inhibiting osteoclast differentiation in vivo.

Thus far, our data show that apigenin antagonizes the effects of TNFα, IFNγ, and RANKL at concentrations that are 10-, 10-, and 25-fold higher than their EC_{50} of 0.2, 0.5, and 2 ng/ml, respectively. The concentration range of apigenin in this study (5–20 μM) in mitigating events stimulated by TNFα, IFNγ, and RANKL is in accordance with the concentrations of various flavonoids used in various other cell types. For example, 25- to 50-μM apigenin inhibited TNFα-induced adhesion of THP-1 monocytes [61], 5 μM resveratrol suppressed TNF-induced activation of the NF-κB pathway in U-937 myeloid cells [62], and 10 μM soy isoflavones or 5–10 μM kaempferol inhibited TNFα-induced IL-6, PGE2, and MCP-1 secretion in osteoblastic cells [63,45]. In the case of IFNγ action, 100 μM silymarin [64] or green tea polyphenol (−)-epigallocatechin gallate (EGCG) [65] were required to inhibit IFNγ-induced nuclear translocation of various signal transducers and activator of transcription (STATs) in RINm5F insulinoma cells or T84 colonic epithelial cells. In the case of RANKL action in RAW 264.7 cells, 3-μM genistein inhibited RANKL-induced NF-κB nuclear localization [66], and 10-μM kaempferol or quercetin inhibited expression of osteoclast differentiation markers [45]. Moreover, the effective concentration of 5–20 μM apigenin in this study was less than half of 40–50 μM apigenin used in most of the studies [67–72]. However, the in vitro concentration we used was 10-fold higher than what could be achieved in animal studies [73]. It is possible that lower in vivo concentrations of apigenin mimic effects observed at much higher concentrations in vitro. For example, whereas 10–40 μM apigenin inhibited TNFα-induced NF-κB activation and induction of apoptosis in PC-3 prostate carcinoma cells in vitro [74], a serum apigenin level of 1.26 μM (used by the same group of investigators in nude mice after 50 μg/mouse/day apigenin treatment) resulted in increased tumor uptake of apigenin with growth inhibition and apoptosis induction in xenograft tumor [73]. Limited bioavailability of apigenin in pure form is a major drawback, and further studies are needed to increase the efficacy of apigenin in in vivo studies [75].

Finally, we studied the effect of apigenin on long bone mature osteoclasts. Our data revealed that apigenin not only inhibited bone resorption by osteoclasts, but also induced osteoclast apoptosis. The apigenin-induced stimulation of apoptosis likely contributes in the inhibition of bone resorption by apigenin. The specificity of apigenin effects has been shown by the lack of effect of flavone at similar concentrations. The failure of apigenin to induce apoptosis in differentiating RAW 264.7 cells while promoting cell death in mature osteoclast could be explained by the presence of RANKL in RAW 264.7 cell culture. RANKL is not only required for the differentiation of osteoclast precursor like RAW 264.7 cells to osteoclasts but it is also the most potent cell survival factor (for review, [76]). Mature osteoclast cultures were devoid of RANKL and therefore represented survival factor withdrawal-induced apoptosis paradigm as previously described [77,78]. Therefore, apigenin appears to affect all stages of osteoclastogenesis, starting from the inhibition of osteoclast differentiation to osteoclast survival and bone resorption. It is tempting to speculate that the combination of the effects of apigenin on osteoblasts, preadipocytes, and osteoclasts might prove useful in the prevention and therapy of bone loss disorders.

Acknowledgements

Funding from National Institutes of Health grants AR02215 to N.C. and ES014462 to J.R.R. is acknowledged.

REFERENCES

[1] Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. Endocr Rev 2002;23:90–119.
[2] Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, et al. Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 1992;257:88–91.
[3] Passeri G, Girasole G, Jilka RL, Manolagas SC. Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. Endocrinology 1993;133:822–8.
[4] Pacifici R, Brown C, Puscheck E, Friedrich E, Slatopolsky E, Maggio D, et al. Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. Proc Natl Acad Sci USA 1991;88:5134–8.

[5] Weitzmann MN, Pacifici R. The role of T lymphocytes in bone metabolism. Immunol Rev 2005;208:154–68.
[6] Aubin JE, Triffitt JT. Mesenchymal stem cells and osteoblast differentiation. In: Bilezikian JP, Raisz LG, Rodan G, editors. Principles of bone biology. 2nd ed, San Diego: Academic Press; 2002. p. 59–81.
[7] Kelly KA, Tanaka S, Baron R, Gimble JM. Murine bone marrow stromally derived BMS2 adipocytes support differentiation and function of osteoclast-like cells in vitro. Endocrinology 1998;139:2092–101.
[8] Botolin S, Faugere MC, Malluche H, Orth M, Meyer R, McCabe LR. Increased bone adiposity and peroxisomal proliferator-activated receptor-gamma2 expression in type I diabetic mice. Endocrinology 2005;146(8): 3622–31.
[9] Zayzafoon M, Gathings WE, McDonald JM. Modeled microgravity inhibits osteogenic differentiation of human mesenchymal stem cells and increases adipogenesis. Endocrinology 2004;145(5):2421–32.
[10] Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T, Kassem M. Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology 2001;2:165–71.
[11] Nuttall ME, Gimble JM. Controlling the balance between osteoblastogenesis and adipogenesis and the consequent therapeutic implications. Curr Opin Pharmacol 2004;4(3):290–4.
[12] Wang B, Jenkins JR, Trayhurn P. Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. Am J Physiol Endocrinol Metab 2005;288(4):E731–40.
[13] Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 1986;319(6053):516–8.
[14] Thomson BM, Mundy GR, Chambers TJ. Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol 1987;138:775–9.
[15] Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, et al. Estrogen deficiency induces bone loss by enhancing T cell production of TNF-alpha. J Clin Invest 2000;106:1229–37.
[16] Roggia C, Gao Y, Cenci S, Weitzmann MN, Toraldo G, Isaia G, et al. Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo. Proc Natl Acad Sci USA 2001;98:13960–5.
[17] Kimble RB, Matayoshi AB, Vannice JL, Kung VT, Williams C, Pacifici R. Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period. Endocrinology 1995;136:3054–61.
[18] Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 1996;11:1043–51.
[19] Ammann P, Rizzoli R, Bonjour JP, Bourrin S, Meyer JM, Vassalli P, et al. Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. J Clin Invest 1997;99:1699–703.
[20] Kimble RB, Bain S, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. J Bone Miner Res 1997;12:935–41.
[21] Mori L, Iselin S, De Libero G, Lesslauer W. Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type 1 (TNFR1)-IgG1-treated and TNFR1-deficient mice. Ann Rheum Dis 1999;58:I40–8.
[22] Keffer J, Probert L, Cazlaris H, Georgopoulos S, Kaslaris E, Kioussis D, et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J 1991;10:4025–31.
[23] Ehlers S. Why does tumor necrosis factor targeted therapy reactivate tuberculosis? J Rheumatol Suppl 2005;74:35–9.
[24] Giles JT, Bathon JM. Serious infections associated with anticytokine therapies in the rheumatic diseases. J Intensive Care Med 2004;19:320–34.
[25] Cush JJ. Unusual toxicities with TNF inhibition: heart failure and drug-induced lupus. Clin Exp Rheumatol 2004;22:S141–7.
[26] Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 1999;96:3540–5.
[27] Yasuda H, Shima N, Nakagawa N. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 1998;95:3597–602.
[28] Hierl T, Borcsok I, Sommer U, Ziegler R, Kasperk C. Regulation of interleukin-6 expression in human osteoblastic cells in vitro. Exp Clin Endocrinol Diabetes 1998;106:324–33.
[29] Zhu JF, Valente AJ, Lorenzo JA, Carnes D, Graves DT. Expression of monocyte chemoattractant protein 1 in human osteoblastic cells stimulated by proinflammatory mediators. J Bone Miner Res 1994;9:1123–30.
[30] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20:345–7.
[31] Baker PJ, Dixon M, Evans RT, Dufour L, Johnson E, Roopenian DC. CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. Infect Immun 1999;67:2804–9.
[32] Cenci S, Toraldo G, Weitzmann MN, Roggia C, Gao Y, Qian WP, et al. Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator. Proc Natl Acad Sci USA 2003;100:10405–10.
[33] Butrimiene I, Jarmalaite S, Ranceva J, Venalis A, Jasiuleviciute L, Zvirbliene A. Different cytokine profiles in patients with chronic and acute reactive arthritis. Rheumatology 2004;43:1300–4.
[34] Kwak HB, Lee SW, Jin HM, Ha H, Lee SH, Takeshita S, et al. Monokine induced by interferon-gamma is induced by receptor activator of nuclear factor kappa B ligand and is involved in osteoclast adhesion and migration. Blood 2005;105:2963–9.
[35] Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Maor G, Karin N. Targeting the function of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. J Immunol 2002;169:2685–93.
[36] Villar A, Gasco MA, Alcaraz MJ. Anti-inflammatory and anti-ulcer properties of hypolaetin-8-glucoside, a novel plant flavonoid. J Pharm Pharmacol 1984;36:820–3.
[37] Gerdin B, Svensjo E. Inhibitory effect of the flavonoid O-(beta-hydroxyethyl)-rutoside on increased microvascular permeability induced by various agents in rat skin. Int J Microcirc Clin Exp 1983;2:39–46.
[38] Agarwal OP. The anti-inflammatory action of nepitrin, a flavonoid. Agents Actions 1982;12:298–302.
[39] Hiremath SP, Badami S, Hunasagatta SK, Patil SB. Antifertility and hormonal properties of flavones of *Striga orobanchioides*. Eur J Pharmacol 2000;391:193–7.
[40] Miksicek RJ. Estrogenic flavonoids: structural requirements for biological activity. Proc Soc Exp Biol Med 1995;208:44–50.

[41] Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewicz-Sapuntzakis M, Guo P, et al. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161–7.
[42] Horcajada-Molteni MN, Crespy V, Coxam V, Davicco MJ, Remesy C, Barlet JP. Rutin inhibits ovariectomy-induced osteopenia in rats. J Bone Miner Res 2000;15:2251–8.
[43] Garcia Palacios V, Robinson LJ, Borysenko CW, Lehmann T, Kalla SE, Blair HC. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 cells by estrogen and phytoestrogens. J Biol Chem 2005;280:13720–7.
[44] Wattel A, Kamel S, Mentaverri R, Lorget F, Prouillet C, Petit JP, et al. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochem Pharmacol 2003;65:35–42.
[45] Pang JL, Ricupero D, Huang S, Fatma N, Singh DP, Romero JR, et al. Differential activity of kaempferol and quercetin in attenuating tumor necrosis factor receptor family signaling in bone cells. Biochem Pharmacol 2006;71:818–26.
[46] Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Butters RR, Brown EM. Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer cell lines. Endocrinology 2000;141(12):4357–64.
[47] Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, et al. Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature 2004;428:758–63.
[48] Foged NT, Delaisse JM, Hou P, Lou H, Sato T, Winding B, et al. Quantification of the collagenolytic activity of isolated osteoclasts by enzyme-linked immunosorbent assay. J Bone Miner Res 1996;11:226–37.
[49] Kameda T, Ishikawa H, Tsutsui T. Detection and characterization of apoptosis in osteoclasts in vitro. Biochem Biophys Res Commun 1995;15(207):753–60.
[50] Yano S, Mentaverri R, Kanuparthi D, Bandyopadhyay S, Rivera A, Brown EM, et al. Functional expression of beta-chemokine receptors in osteoblasts: role of regulated upon activation, normal T cell expressed and secreted (RANTES) in osteoblasts and regulation of its secretion by osteoblasts and osteoclasts. Endocrinology 2005;146:2324–35.
[51] Gitelman SE, Kirk M, Ye JQ, Filvaroff EH, Kahn AJ, Derynck R. Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. Cell Growth Differ 1995;6(7):827–36.
[52] Gruber R, Graninger W, Bobacz K, Watzek G, Erlacher L. BMP-6-induced osteogenic differentiation of mesenchymal cell lines is not modulated by sex steroids and resveratrol. Cytokine 2003;23(4–5):133–7.
[53] Chaudhary LR, Spelsberg TC, Riggs BL. Production of various cytokines by normal human osteoblast-like cells in response to interleukin-1 beta and tumor necrosis factor-alpha: lack of regulation by 17 beta-estradiol. Endocrinology 1992;130(5):2528–34.
[54] Rifas L, Kenney JS, Marcelli M, Pacifici R, Cheng SL, Dawson LL, et al. Production of interleukin-6 in human osteoblasts and human bone marrow stromal cells: evidence that induction by interleukin-1 and tumor necrosis factor-alpha is not regulated by ovarian steroids. Endocrinology 1995;136(9):4056–67.
[55] Garcia-Moreno C, Catalan MP, Ortiz A, Alvarez L, De la Piedra C. Modulation of survival in osteoblasts from postmenopausal women. Bone 2004;35:170–7.
[56] Verbeek R, Plomp AC, van Tol EA, van Noort JM. The flavones luteolin and apigenin inhibit in vitro antigen-specific proliferation and interferon-gamma production by murine and human autoimmune T cells. Biochem Pharmacol 2004;68:621–9.
[57] Takayanagi H, Sato K, Takaoka A, Taniguchi T. Interplay between interferon and other cytokine systems in bone metabolism. Immunol Rev 2005;208:181–93.
[58] Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 2005;115(5):911–9.
[59] Takeda S, Elefteriou F, Karsenty G. Common endocrine control of body weight, reproduction, and bone mass. Annu Rev Nutr 2003;23:403–11.
[60] Yu X, Huang Y, Collin-Osdoby P, Osdoby P. CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. J Bone Miner Res 2004;19:2065–77.
[61] Choi JS, Choi YJ, Park SH, Kang JS, Kang YH. Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule upregulation in cultured human endothelial cells: role of nuclear factor-kappa B. J Nutr 2004;134:1013–9.
[62] Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 2000;164:6509–19.
[63] Suh KS, Koh G, Park CY, Woo JT, Kim SW, Kim JW, et al. Soybean isoflavones inhibit tumor necrosis factor-alpha-induced apoptosis and the production of interleukin-6 and prostaglandin E2 in osteoblastic cells. Phytochemistry 2003;63:209–15.
[64] Matsuda T, Ferreri K, Todorov I, Kuroda Y, Smith CV, Kandeel F, et al. Silymarin protects pancreatic beta-cells against cytokine-mediated toxicity: implication of c-Jun NH2-terminal kinase and janus kinase/signal transducer and activator of transcription pathways. Endocrinology 2005;146:175–85.
[65] Watson JL, Ansari S, Cameron H, Wang A, Akhtar M, McKay DM. Green tea polyphenol (−)-epigallocatechin gallate blocks epithelial barrier dysfunction provoked by IFN-gamma but not by IL-4. Am J Physiol Gastrointest Liver Physiol 2004;287:G954–61.
[66] Garcia Palacios V, Robinson LJ, Borysenko CW, Lehmann T, Kalla SE, Blair HC. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 cells by estrogen and phytoestrogens. J Biol Chem 2005;280:13720–7.
[67] Cotter AA, Cashman KD. The effect of two dietary and a synthetic phytoestrogen on transepithelial calcium transport in human intestinal-like Caco-2 cells. Eur J Nutr 2005;44:72–8.
[68] Fang J, Xia C, Cao Z, Zheng JZ, Reed E, Jiang BH. Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J 2005;19:342–53.
[69] Torkin R, Lavoie JF, Kaplan DR, Yeger H. Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol Cancer Ther 2005;4:1–11.
[70] Zheng PW, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sci 2005;76:1367–79.
[71] Wu K, Yuan LH, Xia W. Inhibitory effects of apigenin on the growth of gastric carcinoma SGC-7901 cells. World J Gastroenterol 2005;11:4461–4.
[72] Kowalski J, Samojedny A, Paul M, Pietsz G, Wilczok T. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1beta and tumor necrosis factor-alpha genes in J774.2 macrophages. Pharmacol Rep 2005;57:390–4.
[73] Shukla S, Mishra A, Fu P, MacLennan GT, Resnick MI, Gupta S. Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. FASEB J 2005;19:2042–4.

[74] Shukla S, Gupta S. Suppression of constitutive and tumor necrosis factor alpha-induced nuclear factor (NF)-kappaB activation and induction of apoptosis by apigenin in human prostate carcinoma PC-3 cells: correlation with down-regulation of NF-kappaB-responsive genes. Clin Cancer Res 2004;10:3169–78.
[75] Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 2002;22:19–34.
[76] Tanaka S, Nakamura K, Takahasi N, Suda T. Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL–RANK signaling system. Immunol Rev 2005;208:30–49.
[77] Park H, Jung YK, Park OJ, Lee YJ, Choi JY, Choi Y. Interaction of Fas ligand and Fas expressed on osteoclast precursors increases osteoclastogenesis. J Immunol 2005;175:7193–201.
[78] Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. Bone 2005;36:840–53.