Quercetin, a potent suppressor of NF-κB and Smad activation in osteoblasts

MASAYOSHI YAMAGUCHI2* and M. NEALE WEITZMANN1,3*

1Atlanta Department of Veterans Affairs Medical Center, Decatur, GA 30033; 2Division of Endocrinology, Metabolism and Lipids, Department of Medicine and 3Winship Cancer Institute, Emory University, Atlanta, GA 30322, USA

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Abstract. Osteoclasts, the bone resorbing cells of the body, form when osteoclast precursors are exposed to the key osteoclastogenic cytokine receptor activator of NF-κB ligand (RANKL), a process requiring induction of NF-κB signaling. Quercetin is a ubiquitous plant-derived flavonoid with well documented anti-inflammatory properties, in part, a consequence of its capacity to downmodulate the NF-κB signal transduction pathway. Consistent with this mechanism of action quercetin is reported to suppress osteoclastogenesis in vitro and prevent bone loss in ovariectomized mice in vivo. By contrast, the effect of quercetin on osteoblasts, the cells responsible for bone formation, is contradictory with conflicting reports of inhibition as well as stimulation. Given our previous reports that NF-κB antagonists promote osteoblast differentiation and activity, we compared the effects of quercetin on osteoclast and osteoblast differentiation and on NF-κB signal transduction in vitro. As expected, quercetin potently suppressed osteoclastogenesis and NF-κB activation induced by RANKL in osteoclast precursors. However, the same doses of quercetin had no effect on osteoblast mineralization, and failed to significantly alleviate the inhibitory effect of NF-κB-induced by TNFα, even though quercetin potently suppressed NF-κB activation in these cells. This apparent contradiction was explained by the fact that addition to its anti-NF-κB activity, quercetin also potently antagonized both TGFβ and BMP-2-induced Smad activation in osteoblast precursors. Taken together our data suggest that multiple competing actions of quercetin mediate both stimulatory and inhibitory actions on osteoblasts with the final physiological effect likely a function of the net balance between these stimulatory and inhibitory effects.

Introduction

Many naturally occurring nutritional factors and chemicals isolated from plants are now under investigation for their medicinal properties. The flavonoid quercetin is one such agent and has been found to possess potent anti-inflammatory effects (1,2), due in part to its anti-NF-κB activity (3). The NF-κB signal transduction pathway is synonymous with inflammation and mediates the effect of multiple inflammatory cytokines including the receptor activator of NF-κB ligand (RANKL) and TNFα, two cytokines that have potent effects on the skeleton. Skeletal deterioration is common in inflammatory states including rheumatoid arthritis and periodontitis and in postmenopausal osteoporosis where estrogen deficiency leads to up-regulation in TNFα and RANKL (4).

At the cellular level osteoclasts, the cells that degrade (resorb) bone are derived from precursors of the monocytic lineage that differentiate into mature osteoclasts under the influence of the key osteoclastogenic cytokine RANKL. TNFα synergizes with RANKL at the level of signal transduction to amplify its osteoclastogenic activity. NF-κB is a major signal transduction pathway for both cytokines and consequently NF-κB activation is necessary to generate osteoclasts and to regulate their function (5-8). Consequently, we have demonstrated that a pharmacological antagonist to NF-κB inhibits osteoclastic bone loss in ovariectomized mice in vivo (9) and that natural NF-κB antagonists including vitamin K2 (10) and 17β-estradiol (11) suppress osteoclastogenesis in vitro. NF-κB antagonism has also been shown to ameliorate bone degradation in an animal model of rheumatoid arthritis (12).

Studies have reported that quercetin antagonizes bone resorption in femoral organ cultures (13) and in osteoclast differentiation and/or activation assays in vitro (14,15) by a mechanism that involves suppression of NF-κB and AP-1 (16). Interestingly, a quercetin-like variant, quercetin-6-C-β-D-glucopyranoside isolated from Ulmus wallichiana planchon has been demonstrated to possess enhanced anti-osteoclastogenic activity and to mitigate ovariectomy-induced bone loss in rats (17).

In contrast to osteoclasts, osteoblasts, the cells that build and regenerate bone are potently inhibited by TNFα (18). Furthermore, we recently demonstrated that mice deficient in TNFα or its type I receptor achieve a significantly higher bone mineral density as a consequence of elevated bone formation in vivo (19). We further demonstrated that pharmacological

*Contributed equally

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antagonists of NF-κB activation promote osteoblast differentiation and mineralization in vitro (10,19,20). Recently our studies were ratified in vivo by a report of time- and stage-specific inhibition of NF-κB activation in differentiated osteoblasts leading to increased trabecular bone mass and bone mineral density (21). Additional studies have reported increased bone formation and amelioration of osteopenia in ovariectomized mice treated with pharmacological NF-κB inhibitors (22). Also consistent with these data TNFα antagonists have been shown to promote bone regeneration in a fracture repair model (23).

The action of quercetin on osteoblasts and bone formation however is contentious. Quercetin has been reported to inhibit the proliferation, differentiation, and mineralization of osteoblasts in vitro (24) and promote TNFα-induced inhibition (25) and apoptosis (25,26). Other studies however, report increased alkaline phosphatase (an early osteoblast differentiation marker) levels in human MG-63 osteoblast cells (27) and stimulatory effects on bone formation in rat organ cultures ex vivo (13).

In this study we compared the effects of quercetin on osteoclastogenesis and on osteoblast differentiation. Our data reveal that despite potent suppression by quercetin of TNFα-induced NF-κB in osteoblasts, the overall effects of quercetin on osteoblast differentiation were not stimulatory, a possible consequence of other non-specific actions of quercetin on other pathways including direct suppressive actions of quercetin on Smad signal transduction.

Materials and methods

Materials. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone. RANKL, TNFα, TGFβ and BMP-2 were from R&D Systems (Minneapolis, MN). Quercetin, anti-poly-histidine antibody, leukocyte acid phosphatase kits and all other reagents were purchased from the Sigma-Aldrich Chemical Corporation, (St. Louis, MO) unless otherwise specified.

Cell culture. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) was purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described (19,28).

Osteoclastogenesis assays and TRAP staining. RAW264.7 cells cultured for 6 days with RANKL (30 ng/ml) pre-incubated for 10 min with crosslinking anti-poly-histidine antibody (2.5 µg/ml) to induce osteoclast formation, in the presence or absence of quercetin at the indicated dosage. Cells were fixed and stained for tartrate resistant acid phosphatase (TRAP) activity using a leukocyte acid phosphatase kit. TRAP+ cells with three or more nuclei were defined as osteoclasts and were quantitated under light microscopy and 5 wells/group were averaged.

Osteoblast differentiation assays and Alizarin Red-S staining. Mineralization assays using MC3T3 cells were performed in the presence or absence of quercetin at the indicated dosage, with calcium visualization using Alizarin Red-S. Experimental procedures were performed exactly as described in our previous studies (10,19,29).

NF-κB constructs and luciferase assays. NF-κB and Smad activation was assessed in MC3T3 or RAW264.7 cells using luciferase reported assays using the NF-κB responsive reporter pNF-κB-Luc (BD Biosciences) or pGL3-Smad, responsive to all receptor-regulated (R)-Smads as previously described (10,19). Some cultures were treated with quercetin at the indicated dosages.

Statistical analysis. Statistical significance was determined using the GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test for parametric data. The Gaussian distribution was assessed using the Kolmogorov and Smirnov test. A P-value <0.05 was considered statistically significant.

Results

Quercetin potently suppresses RANKL-induced osteoclast formation. The effect of quercetin on osteoclast formation was examined using RAW264.7 cells which differentiate into mature multinucleated osteoclasts when treated with RANKL. Quercetin potently and dose-dependently inhibited RANKL-induced osteoclastogenesis in the examined range of concentrations (0.1, 1, 10, and 25 µM) (Fig. 1A).

Figure 1. Quercetin suppresses osteoclast differentiation and RANKL-induced NF-κB activation in vitro. (A) RAW264.7 cells were treated with RANKL (30 ng/ml) in the presence or absence of a dose range of quercetin (0, 0.1, 1, 10 or 25 µM) for 7 days and TRAP stained. TRAP+ multinucleated cells (3 or more nuclei) were quantitated and averaged for 5 independent wells for each data point. *P<0.001 relative to RANKL only (grey bar). (B) RAW264.7 cells were transfected with NF-κB-Luc reporter and NF-κB activation induced by stimulation with RANKL (30 ng/ml), in the presence or absence of a dose range of quercetin (0, 0.1, 1, 10 or 50 µM). Data represent the mean ± SD of 5 replicate samples. All data are representative of 2 independent experiments, +P<0.05 and *P<0.001 vs. RANKL only (grey bar).
Figure 2. Effect of quercetin on osteoblast mineralization under basal and TNFα-stimulated conditions in vitro. MC3T3 cells were cultured in the presence (+) or absence (-) of mineralizing medium (MM) and with a dose range of quercetin (0, 0.1, 1, 10 or 50 µM). The effects of quercetin on mineralization were assessed under: (A) basal conditions or (B) in the presence or absence of TNFα (5 ng/ml). Cells were stained for calcium deposition with Alizarin Red-S. For each experiment all wells were performed in duplicate and are derived from the same plate but were digitally rearranged for clarity. All data are representative of 2 independent experiments.

To assess the effect of quercetin on NF-κB activation in osteoclasts we transfected an NF-κB luciferase reporter into RAW264.7 cells and measured luciferase activity 18 h later. Consistent with the osteoclastogenesis assays quercetin antagonized RANKL-induced NF-κB activation in the dose range examined (0.1, 1, 10 and 50 µM) (Fig. 1B).

Quercetin has no effect on the differentiation of MC3T3 cells into mineralizing osteoblasts at a low-dose but inhibits mineralization at a high-dose. In contrast to osteoclasts in which quercetin is generally found to be inhibitory, the data for osteoblast differentiation is contradictory. Given the known inhibitory action of NF-κB on osteoblast differentiation and the anti-NF-κB activity of quercetin it would be expected for quercetin to have a stimulatory role in osteoblasts. The effect of quercetin on in vitro osteoblast differentiation and activity was assessed by treating MC3T3 cells with quercetin at (0.1, 1, 10 or 50 µM) in mineralizing medium for 21 days followed by Alizarin Red-S staining for calcium deposition. The data demonstrate (Fig. 2A) that between 0.1 and 10 µM quercetin had no effect on mineralization, but at 50 µM had a suppressive activity on the differentiation of MC3T3 cells into mineralizing osteoblasts.

TNFα is a potent inhibitor of osteoblastic differentiation both in vivo and in vitro (18,19) but this repression can be alleviated by NF-κB antagonists. We thus examined the effect of quercetin on TNFα (5 ng/ml)-induced osteoblast mineralization. As expected TNFα inhibited mineralization (Fig. 2B). Quercetin at 0.1 and 1 µM exhibited a very weak alleviation of TNFα's inhibitory action, but by 10 µM slightly exacerbated the inhibitory effect of TNFα.

Quercetin suppresses basal and TNFα-induced NF-κB activity in MC3T3 cells. Because quercetin is reported to antagonize NF-κB, a pathway inhibitory to osteoblast differentiation and a key mechanism of TNFα signaling we next investigated whether quercetin antagonizes basal and/or TNFα-induced NF-κB activity. MC3T3 cells were transiently transfected with an NF-κB luciferase reporter and we quantitated luciferase activity in the presence or absence of quercetin 18 h later. Quercetin potently and dose-dependently suppressed both basal and TNFα-induced NF-κB activity (Fig. 3).

Quercetin fails to alleviate the suppressive actions of TNFα on BMP-2- and TGFβ-induced Smad activation in MC3T3 cells and directly inhibits TGFβ and BMP-2-induced Smad activation. TGFβ and BMP-2 are early and late osteoblast commitment and differentiation factors respectively, and both signal in a large measure though the Smad pathway, albeit through different R-Smads. The effect of quercetin on BMP-2-induced (Fig. 4A) and TGFβ-induced (Fig. 5A) Smad activation was assessed in MC3T3 cells transfected with a Smad-luciferase reporter (19). Surprisingly, quercetin alone elicited a direct potent dose responsive (0.1 to 25 µM) suppression of both BMP-2-induced (Fig. 4A) and TGFβ-induced (Fig. 5A) Smad activation.

We have previously shown that TNFα suppresses osteoblast differentiation, in part, by antagonizing Smad activation by BMP-2 and TGFβ. Consequently, we examined the capacity of quercetin to alleviate the suppressive action of TNFα on Smad induction by BMP-2 and TGFβ. Quercetin failed to alleviate the suppressive action of TNFα on BMP-2-induced (Fig. 4B), or TGFβ-induced (Fig. 5B) Smad-activation. In fact, quercetin significantly amplified the inhibitory effect of TNFα on Smad activation induced by both cytokines.

Discussion

Quercetin has been previously reported to suppress osteoclast differentiation (13,14,16), in part by antagonizing the NF-κB pathway.
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Our data are fully consistent with these previous studies.

By contrast, the effect of quercetin on osteoblast differentiation is less clear cut with both suppression and stimulation being reported (13,24-27,30). As we have previously demonstrated that a number of factors with bone anabolic activity function by antagonizing NF-κB (10,11,19,20), we assessed the effect of quercetin on NF-κB activation in MC3T3 osteoblast precursors. While our data indeed validate a potent suppressive effect of quercetin on TNFα-induced NF-κB activation, surprisingly this did not translate into a strong bone anabolic effect. Instead quercetin displayed very weak anabolic activity at the low-dose and even a suppressive activity at high-dose, although general toxicity was not observed.

We have previously shown that TNFα is a potent physiological and pathological suppressor of bone formation and reduces peak bone mineral density in vivo (19). One mechanism by which TNFα suppresses osteoblastogenesis is by antagonizing the activation of Smad signal transduction by TGFβ and BMP-2 (19), early commitment (31) and late differentiation (32) factors respectively. We have further demonstrated that NF-κB antagonists generally reverse the suppressive action of TNFα on Smad signaling (10,19,20). In the case of quercetin this outcome was not found to be true and quercetin significantly added to the inhibitory action of TNFα, rather than alleviating it. Although unexpected, our data are consistent with a previous report in which quercetin inhibited Smad activation in keloid-derived fibroblasts (33).

Taken together our data suggest the existence of complex competing actions of quercetin on osteoblast signal transduction pathways. Ultimately, the net effect of quercetin on osteoblasts in vitro and in vivo may be dose and context dependent with the prevailing concentrations of competing cytokines and growth factors in the bone marrow microenvironment determining whether the final effect is stimulatory or inhibitory to bone formation.

The specific mechanisms by which quercetin is able to modulate both NF-κB and Smad, and potential other signaling systems remains to be determined, and may ultimately facilitate the rational design of antagonists possessing either NF-κB or Smad inhibitory actions for use as anti-inflammatory pharmaceuticals to ameliorate bone loss and other diseases associated with inflammation.

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