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Flavonoid Quercetin Decreases Osteoclastic Differentiation Induced by RANKL via a Mechanism Involving NFκB and AP-1

Alice Wattel,[1]* Said Kamel,[1] Christophe Prouillet,[1] Jean-Pierre Petit,[1] Florence Lorget,[2] Elizabeth Offord,[2] and Michel Brazier[1]

[1]Unité de Recherche sur les Mécanismes de la Résorption Osseuse, Laboratoire de Pharmacie Clinique, Université de Picardie Jules Verne, 1 rue des Louvels, 80037 Amiens, France
[2]Department of Nutrition, Nestlé Research Center, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland

Abstract Flavonoids are micronutrients widely present in food of plant origin. They have been attributed pharmacological properties such as anticancer and prevention of age-related pathologies. It has been recently hypothesized that flavonoids increase bone mass and prevent osteoporosis. However, little is known about the in vitro effects of flavonoids on osteoclast activities. We investigated the effects of quercetin, one of the most commonly occurring flavonoids, on osteoclast differentiation which is a critical determinant step of in vivo bone resorption. Two in vitro models of osteoclast differentiation were used in this study: a murine one, involving the culture of RAW 264.7 cells in presence of receptor activator of NFκB ligand (RANKL), and a human model consisting of differentiating peripheral blood monocytic cells (PBMC) isolated from peripheral blood in presence of RANKL and macrophage-colony stimulating factor (M-CSF). Osteoclastogenesis was assessed by osteoclast-like number, tartrate resistant acid phosphatase (TRAP) activity, and bone resorbing activity. We showed that quercetin (0.1–10 μM) decreased osteoclastogenesis in a dose dependent manner in both models with significant effects observed at low concentrations, from 1 to 5 μM. The IC_{50} value was about 1 μM. Analysis of protein–DNA interaction by electrophoretic mobility shift assay (EMSA) performed on RAW cells showed that a pre-treatment with quercetin inhibited RANKL-induced nuclear factor kB (NFκB) and activator protein 1 (AP-1) activation. NFκB and AP-1 are transcription factors highly involved in osteoclastic differentiation and their inhibition could play an important role in the decrease of osteoclastogenesis observed in the presence of quercetin. In conclusion, the present results demonstrate for the first time that quercetin, a flavonoid characterized by antioxidant activities, is a potent inhibitor of in vitro osteoclastic differentiation, via a mechanism involving NFκB and AP-1. J. Cell. Biochem. 92: 285–295, 2004.

Key words: osteoclast; differentiation; bone resorption; flavonoids; quercetin; NFκB; AP-1

Osteoclast differentiation is a central event in regulation of bone resorption process. Numerous pharmacological substances used in osteoporosis prevention are known to decrease in vivo the number of mature osteoclasts formed from the hematopoietic lineage colony forming unit-granulocyte macrophage (CFU-GM). Nowadays, it is of growing interest to promote a nutritional strategy to prevent the decrease of bone mass and the development of osteoporosis. Indeed, recent epidemiological [Tucker et al., 1999; Hegarty et al., 2000; New et al., 2000] and experimental [Muhlbauer and Li, 1999; Muhlbauer et al., 2002] studies have demonstrated a positive effect of consumption of fruits, vegetables, and beverages such as tea on bone mineral density (BMD), suggesting that plant derived foodstuffs may contain bioactive molecules able to modulate bone metabolism. Fruits, vegetables, and tea are a major source of flavonoids, which represent a family of natural substances described as having numerous biochemical properties like antioxidant and

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*Correspondence to: Alice Wattel, Unité de Recherche sur les Mécanismes de la Résorption Osseuse, Laboratoire de Pharmacie Clinique, Université de Picardie Jules Verne, 1 rue des Louvels, 80037 Amiens, France.
E-mail: alice.wattel@sa.u-picardie.fr

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estrogenic effects [Nijveldt et al., 2001]. Flavonoids are mostly present in the form of glycosides in vegetables and fruits. After ingestion, aglycone forms are generated and they are accumulated as glucuronide and sulfate conjugates in circulation [Moon et al., 2000]. In the bone field, soybean isoflavones, a subclass of flavonoids mainly represented by genistein and daidzein, have received considerable attention for their potential role in preventing postmenopausal bone loss [Anderson and Garner, 1998; Kimira et al., 1998; Setchell and Cassidy, 1999; Morabito et al., 2002]. Numerous in vitro studies have been yet published about the effects of isoflavones on bone cells, and it has been recently shown that genistein and daidzein decreased osteoclast differentiation [Yamagishi et al., 2001; Rassi et al., 2002]. Interestingly, it has been suggested that other subfamilies like citrus flavonoids [Chiba et al., 2003] and flavonols [Horcajada-Molteni et al., 2000] may have beneficial effect on bone health but very few studies have been conducted to gain insight into their possible mechanism of action.

In a previous work, we demonstrated that quercetin (3,3′,4′,5,7-pentahydroxyflavone), the primary dietary flavonol, exerts a potent inhibitory effect on the in vitro bone resorbing activity of mature osteoclasts by inducing their apoptosis [Wattel et al., 2003]. Thus, the aim of the present study was to investigate the effects of quercetin on osteoclastogenesis. For this purpose, we used two in vitro osteoclastogenesis systems: firstly, a human model in which osteoclastic cells were differentiated from human peripheral blood monocytic cells (PBMC) under stimulation by receptor activator of NFκB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), and, secondly, a model consisting in differentiating osteoclast-like (OCLs) from the murine monocytic-macrophagic RAW 264.7 cells in presence of RANKL. OCL formation was assessed by counting the number of tartrate resistant acid phosphatase (TRAP)-positive multinucleated cells, by measurement of TRAP activity and also bone resorption activity. Induction of osteoclastic differentiation by RANKL requires the activation of nuclear factor kB (NFκB) and activator protein 1 (AP-1) transcription factors via tumor necrosis associated factors (TRAF) family proteins from receptor activator of NFκB (RANK) [Darnay et al., 1998; Ye et al., 2002]. Mice lacking NFκB and c-fos, a subunit of AP-1, lead to a defect in osteoclast differentiation and to an osteopetrosis, demonstrating that NFκB and AP-1 are two key transcription factors highly involved in osteoclastic differentiation [Grigoriadis et al., 1994; Iotsova et al., 1997]. We thus evaluated the regulatory effect of quercetin on NFκB and AP-1 by electrophoretic mobility shift assay (EMSA) using RAW 264.7 cells nuclear extracts. The results presented here demonstrate for the first time that quercetin significantly decreases osteoclast differentiation at concentrations as low as 1 μM by a mechanism involving at least in part the inhibition of NFκB and AP-1 activation.

MATERIALS AND METHODS

Reagents

Culture media RPMI and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma (St. Quentin Fallavier, France) and αMEM without phenol red was from Life Technologies (Cergy Pontoise, France). All media were supplemented with 1% glutamine (Sigma) and 1% penicillin–streptomycin (Sigma). Fetal calf serum (FCS) was obtained from Dominique Dutscher (Issy-Les-Moulineaux, France). Human RANKL was obtained from Alexis Biochemicals (Paris, France), human M-CSF was from R&D Systems (Lille, France), and murine RANKL was from Tebu-bio (Le Perray en Yvelines, France). Quercetin (3,5,7,3′,4′-pentahydroxyflavone), phosphate-buffered saline (PBS), and all other reactives were purchased from Sigma. Quercetin was dissolved in dimethyl sulfoxide (DMSO, Sigma). In all experiments, respective controls were treated with vehicle. In control or test media, DMSO concentration never exceeded 0.05%.

Differentiation of PBMC and RAW 264.7 Cells In Vitro

PBMC. Whole blood was provided by the Blood Transfusion Center of Amiens and human PBMC were isolated from peripheral blood using Histopaque® density gradient centrifugation (density = 1.077). Isolated cells were plated in 48-well plates at a density of 5×10^5 cells per well, to a final volume of 300 μl of RPMI containing 10% FCS. Bovine bone slices (6 mm of diameter) were previously placed into these wells. After 2 h of incubation in RPMI 10% FCS, we removed the media, washed the bone slices to eliminate the non-adherent cells, and added αMEM medium supplemented with 10% of FCS,

human RANKL (25 ng/ml) and human M-CSF (30 mg/ml). All media and added factors were replaced twice weekly for 14 days of culture.

RAW 264.7. RAW 264.7 mouse macrophage/monocytes were purchased from American Type Culture Collection (TIB-71, Molsheim, France). Cells were cultured routinely in DMEM containing 10% heat-inactivated FCS. In order to differentiate these cells in OCLs, RAW cells were gently scraped and seeded in 96-well plates at a density of 10^3 cells/well. After 2 h, media were changed and replaced by αMEM without phenol red supplemented with 10% FCS and 30 ng/ml recombinant murine RANKL. Media were replaced at the third day and cells were cultured for 5 days. The ability of RAW 264.7 cell-derived osteoclasts to resorb bone was assessed both on bovine bone slices and on plates coated with calcium-phosphate apatite (OAAS®, Osteogenic Core Technologies, Torrance, CA) with RANKL (30 ng/ml) for 10 days.

Cytochemical Staining

Cells were stained for TRAP using a commercially available kit. Briefly, cells were washed with PBS and fixed in formaldehyde 3.7% in PBS for 10 min. Then, the cells were incubated at 37°C in staining solution and rinsed twice in PBS. Multinucleated (>3 nuclei) TRAP-positive cells (MNCs) were counted as osteoclasts under microscopic examination.

TRAP Activity Measurement

After 5 days of culture, RAW 264.7-derived osteoclasts were lysed and incubated for 1 h with a reaction buffer containing paranitrophenylphosphate (pNPP). The reaction was stopped with NaOH 0.3 N and optical densities (ODs) were read using a microplate spectrophotometer at 405 nm. ODs were compared to a standard curve calibrated with paranitrophenol (pNP). Protein contents were quantified with Biorad Protein Assay, using serum albumin as standard. Results were expressed as micromoles of pNP/mg protein.

Assessment of Bone Resorption

Pit area measurement. The PBMC-derived adherent cells were removed from the bone slices and the slices were stained with toluidine blue 1%, allowing the visualization of resorption lacunae or pits. The percentage of bone surface resorbed was quantified by a computerized method (Software Biocom Countscan®).

Biochemical determination. The hydroxylysylpyridinoline (HP) content was determined by high-performance liquid chromatography (HPLC) according to a method previously described [Lorget et al., 2000]. Briefly, supernatants of PBMC cultures were pooled and stored at −20°C. Each sample subsequently underwent an acid hydrolysis for 20 h at 105°C, followed by an extraction using F1 cellulose column. The resulting eluate was freeze-dried. HP was quantified by fluorescence of the eluate peak using a Jasco FP1520 spectrofluorimeter with excitation at 297 nm and emission at 380 nm. The amount of HP was calculated in nanomolar per well and expressed as percentage of control.

MTT Assay

Cytotoxicity of quercetin against RAW 264.7 cells was measured by the colorimetric assay MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) according to standard protocol. This test is based on the capacity of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring and to generate purple formazan crystals insoluble in aqueous solutions. Experiments were performed in 24-well plates and RAW 264.7 cells were grown at a density of 4×10^3 cells per well in 10% FCS medium containing different concentrations of quercetin for 5 days. Briefly, media were replaced at day 3, and at the end of the culture, 40 μl of MTT solution (5 mg/ml) were added per well and incubated at 37°C for further 4 h. Then media were removed and solvent (0.1 N HCl isopropanol) was added to dissolve the reduced MTT crystals (formazan) present in cells. The OD was measured on a spectrophotometer at a wavelength of 570 nm for reading and 660 nm for the reference. Results are given as a percentage of control.

EMSA

Nuclear protein cell extract. RAW 264.7 cells were grown in culture dishes. After treatment, cells were washed, scraped in ice PBS, and transferred to microcentrifuge tubes to be centrifuged for 3 min at 2,000 rpm. Cell pellets were resuspended in 100 μl of lysis buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PEFABloc®, and 0.5% Nonidet P40) and incubated on ice for 10 min. The cytoplasmic extracts were removed by centrifugation (20 min, 12,000 rpm). The pelleted nuclei were resuspended in 50 μl of

buffer C (20 mM HEPES pH 7.9, 0.5 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PEFABloc®) and incubated on ice for 30 min. The supernatants (nuclear extracts) were obtained after centrifugation (30 min, 15,000 rpm) and quick-frozen at −80°C. Protein content was quantified by the Biorad Protein Assay (Bradford method).

Electrophoresis. Extracts (10 μg) were incubated for 20 min with 1 μg of poly(dI-dC) in binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, and 25% glycerol), and ^{32}P-(ATP)-end labeled NFκB double stranded oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or ^{32}P-(ATP)-end labeled AP-1 double stranded oligonucleotide (5′-CGCTTGATGACTCAGGGGGAA-3′). DNA binding was separated from the free probe using 5% polyacrylamide–bisacrylamide 29:1 in 0.5 × TBE running buffer for 2.5 h at 150 V. Gels were dried and the DNA–protein complex was visualized by autoradiography.

Statistical Analysis

The results were expressed as mean ± SEM. Three independent experiments (n = 4 for each experiment) have been performed, except for HP quantification (one experiment with n = 4) and MTT assay (two experiments with n = 4 for each experiment). Statistical significance was determined by Mann–Whitney's test and a value of $P < 0.05$ was considered as significant.

RESULTS

Characterization of OCLs in Murine and Human Models

Murine RANKL (30 ng/ml) alone induced the formation of numerous multinucleated TRAP-positive cells from RAW 264.7 cells after 5 days of culture in 96-well plates (Fig. 1a). Approximately 250 OCLs were counted per control well. These cells present morphological features of mature osteoclasts: multinucleation and TRAP-positive staining. Biochemical TRAP activity measurement confirmed the presence of TRAP in RAW cells cultured with RANKL as compared to undifferentiated cells (Fig. 1d). Another important characteristic of these cells is their ability to resorb bone. RAW cells were cultured on bovine bone slices and on synthetic hydroxyapatite matrix (OAAS®). After 10 days of culture, we observed between 5 and 10 resorption pits on both bone slices and OAAS® slides (Fig. 1b,c). Control cultures were unable to form resorption pits.

After 14 days of culture in the presence of human soluble M-CSF (30 ng/ml) and RANKL (25 ng/ml), PBMC differentiated in TRAP-positive multinucleated cells as shown on Figure 1e. These cells demonstrated a very high capacity to make lacunae in bone matrix (Fig. 1f). The relationship between OCLs number and resorbed bone area showed a correlation with a coefficient r = 0.9 (Fig. 1g).

Effects of Quercetin on Osteoclastogenesis

The effects of quercetin (0.1–10 μM) on the differentiation of RAW 264.7 cells in OCLs are represented in Figure 2. TRAP-positive multinucleated cell formation was strongly inhibited by quercetin in a dose-dependent manner. Significant differences were observed at concentrations as low as 1 μM and the IC_{50} (inhibitory concentration of 50% of basal differentiation) was evaluated to 1.5 μM (Fig. 2a).

TRAP activity measured in the RAW cells after 5 days of treatment with various concentrations of quercetin is shown in Figure 2b. TRAP activity decreased in the presence of the flavonoid in a dose-dependent manner and significant values were obtained at a concentration of 5 μM.

Quercetin also showed a potent inhibitory effect on osteoclast differentiation in the human model. Quercetin significantly decreased the number of OCLs formed (Fig. 3a) and the bone area resorbed by these cells in a dose dependent manner (Fig. 3b). The IC_{50} were respectively 1.4 and 1.05 μM. An inhibition of bone resorption by quercetin was also assessed by a dose-dependent decrease of HP, a collagen cross-linked molecule released in culture supernatants (Fig. 3c).

Evaluation of Toxic Effects of Quercetin on RAW 264.7 Cells

Cytotoxicity of quercetin (0.1–10 μM) was determined by the MTT assay. After 5 days of culture, 10 μM of quercetin slightly decreased cell proliferation of RAW cells. At lower concentrations, this compound did not affect RAW cell proliferation (Fig. 4).

Effects of Quercetin on Transcription Factors NFκB and AP-1 in RAW 264.7 Cells

Because RAW 264.7 represent a cell model with an homogeneous population of osteoclastic

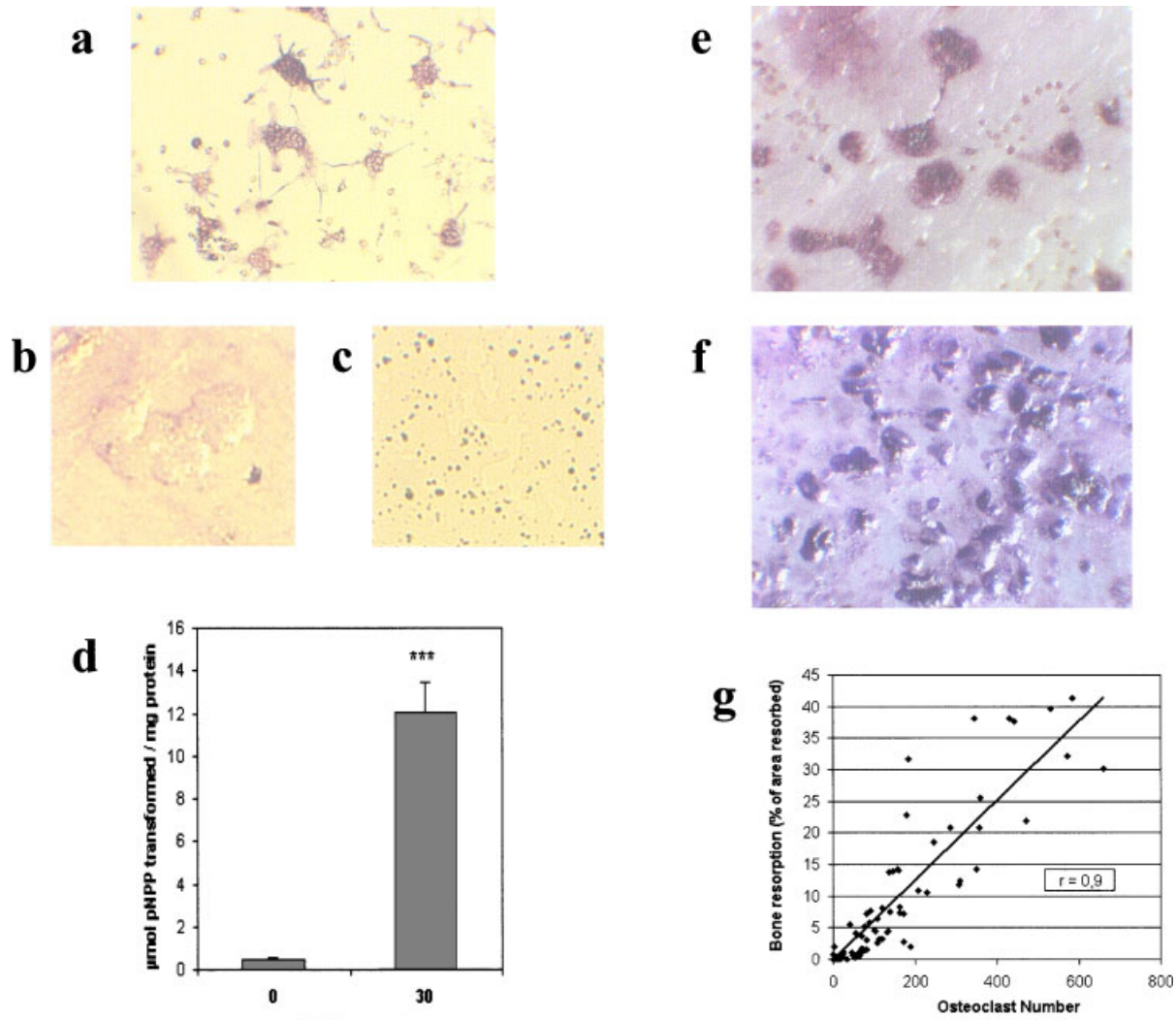

Fig. 1. Characterization of osteoclast-like (OCLs) differentiated from murine RAW 264.7 cells and human PBMC. RAW 264.7 cells were incubated for 5 days in the presence of murine RANKL (30 ng/ml) in a α-MEM medium. (**a**) TRAP staining shows formation of multinucleated TRAP-positive cells when RANKL is added to culture medium. Bone resorbing capacity of RAW 264.7-derived OCLs was confirmed on bone slice (**b**) and on synthetic hydroxyapatite matrix (OAAS®) (**c**) after 10 days of culture in presence of RANKL. (**d**) TRAP activity measurement confirmed the presence of TRAP enzyme in cells cultured in presence of RANKL. PBMC were cultured for 14 days in presence of human RANKL (25 ng/mL) and M-CSF (30 ng/mL) on bovine bone slices. (**e**) TRAP staining shows multinucleated TRAP-positive cells. (**f**) Toluidine blue staining on bone slices reveals a lot of resorption lacunae. (**g**) Relationships between the number of OCLs present on the bone slice at the end of the culture and the percentage of resorption evaluated on this slice. A linear regression was established and the coefficient of correlation calculated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

precursors, we chose to study the effect of quercetin on NFκB and AP-1 on this cell line, rather than on PBMC which represent an heterogeneous cellular population.

Specificity of the shifted bands for NFκB was checked by competition test with a cold oligonucleotide added in excess and with a cold oligonucleotide containing a slightly mutated NFκB site (5′-AGTTGAGGCGACTTTCCCAGGC-3′) (data not shown). Supershifts with the antibodies anti p65 and anti p50 were also performed to determine whether the shifted band corresponded to a p50–p65 heterodimer (data not shown).

RANKL (30 ng/ml) increased NFκB activation after 30 min of incubation: NFκB activation is greatly increased in cells cultured in presence of RANKL (Fig. 5a, lane 2). Therefore, we

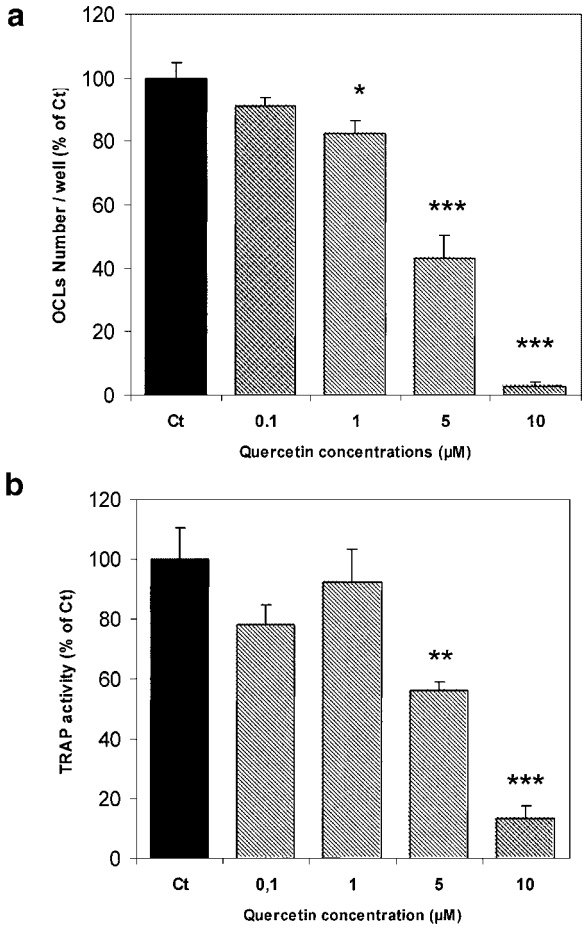

Fig. 2. Effect of quercetin (0.1–10 μM) on osteoclast formation in murine RAW 264.7 cells. OCLs were differentiated from RAW cells cultured for 5 days in presence of murine RANKL (30 ng/ml) (**a**) effect of quercetin on multinucleated TRAP-positive OCLs number per well. **b**: Dose response of TRAP activity to quercetin in OCLs. TRAP activity was expressed as percentage of control. Values are the mean ± SEM of three independent experiments (n = 4 for each experiment). $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) compared with control group.

investigated the effect of quercetin on RANKL-stimulated NFκB activation. Preincubation for 2 h of the cells with different concentrations of flavonoids before the addition of RANKL (30 ng/ml) for 30 min showed quercetin tested at 10, 1, and 0.1 μM dramatically prevented the activation of NFκB induced by RANKL (Fig. 5a, lanes 3–5).

The shifted band obtained with AP-1 oligonucleotide was characterized by competition with the "cold" unlabeled oligonucleotide in excess and with a mutated one (5′-CGCTTGATGTGGCCGGAA-3′) (data not shown). RANKL induced a slight activation of AP-1 after 30 min of incubation (Fig. 5b, lane 2). Preincubation of RAW cells with quercetin at 0.1 μM did not have any effect on RANKL-induced AP-1 activation (Fig. 5b, lane 5). However, quercetin at 1 μM and especially at 10 μM decreased AP-1 activation (Fig. 5b, lanes 3–4).

DISCUSSION

Dietary habits might play a fundamental role in the prevention of osteoporosis. However, except for the possible role of calcium and vitamin D intake, our understanding of the influence of nutrition on bone health remains largely unknown. Recent studies have focused on the potential role of fruit, vegetable, and tea intakes on bone metabolism [Tucker et al., 1999; Hegarty et al., 2000; New et al., 2000]. These results have been extended with the demonstration that common foodstuffs of plant origin inhibit bone resorption in the rat [Muhlbauer and Li, 1999; Muhlbauer et al., 2002].

Herein, we reported several lines of evidence that quercetin, the most widespread naturally occurring flavonol regularly consumed by humans, is a potent in vitro inhibitor of osteoclastogenesis. Our study was conducted using two cellular models widely accepted to investigate the process of osteoclastic differentiation which is a critical determinant step of in vivo bone resorption. As already described, the murine macrophagic-monocytic cell line RAW 264.7 is able to differentiate into OCLs [Shevde et al., 2000; Quinn et al., 2001; Srivastava et al., 2001; Battaglino et al., 2002; Hotokezaka et al., 2002] and does not require M-CSF [Hsu et al., 1999]. We showed that the multinuclear TRAP-positive cells possessed major characteristics of osteoclasts: multinucleation, presence of TRAP, and a slight bone resorbing capacity. Taken together, these results prove that OCLs derived from RAW 264.7 are functionally equivalent to authentic osteoclasts. However, they do not constitute a good model for bone resorption assessment because of their too low resorptive capacity. Human PBMC have been shown to contain osteoclastic precursors (CD14-positive cells) [Matsuzaki et al., 1998; Shalhoub et al., 2000; Lorget et al., 2002] and OCLs differentiated from PBMC are able to form a lot of resorption pits. Osteoclast differentiation was evaluated by different methods according to the model: OCL number and TRAP activity in RAW model, and bone resorption and OCL number in PBMC model. In this last one, the study of correlation between these two parameters leads to a very strong relationship

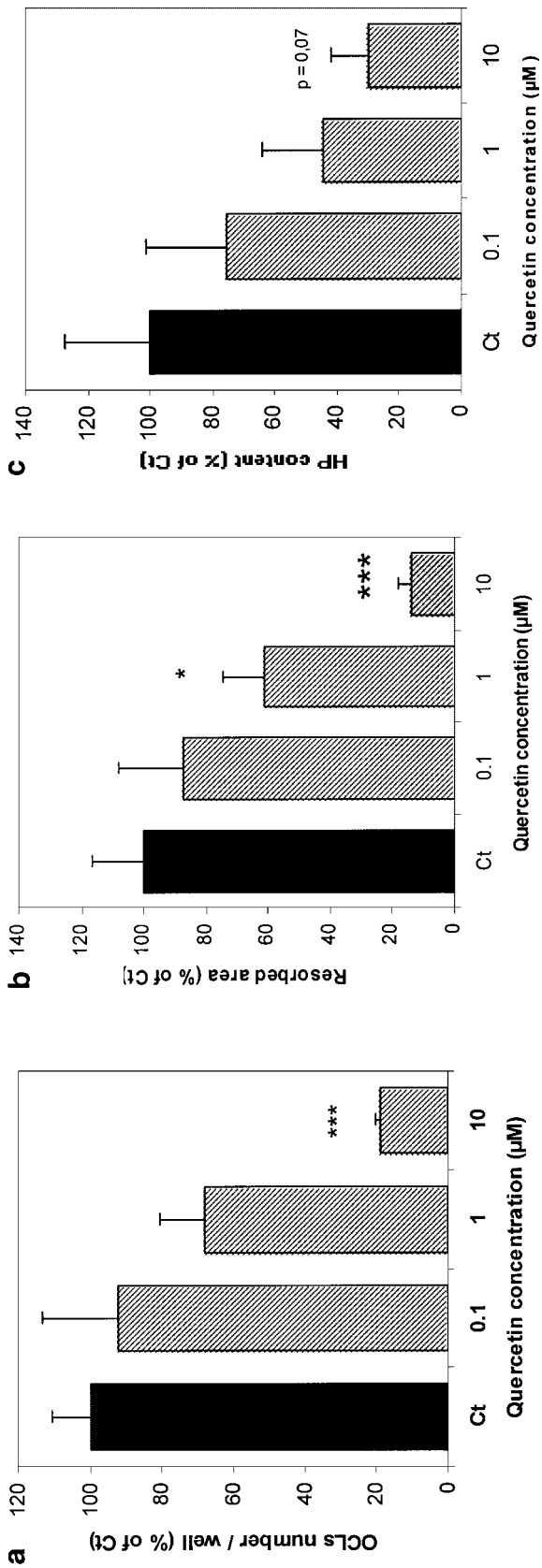

Fig. 3. Effect of quercetin (0.1–10 µM) on osteoclast formation in human model. PBMC were cultured for 14 days on bovine bone slices with human RANKL (25 ng/ml) and M-CSF (30 ng/ml) and in presence of different concentrations of quercetin. **a**: Effect of quercetin on multinucleated TRAP-positive cells number. **b**: Effect of quercetin on bone area resorbed. Values are the mean ± SEM of three independent experiments ($n = 4$ for each experiment). $P < 0.05$ (*) and $P < 0.001$ (***) compared with control group. **c**: Effect of quercetin on hydroxylysylpyridinoline (HP) released in culture supernatants ($n = 4$).

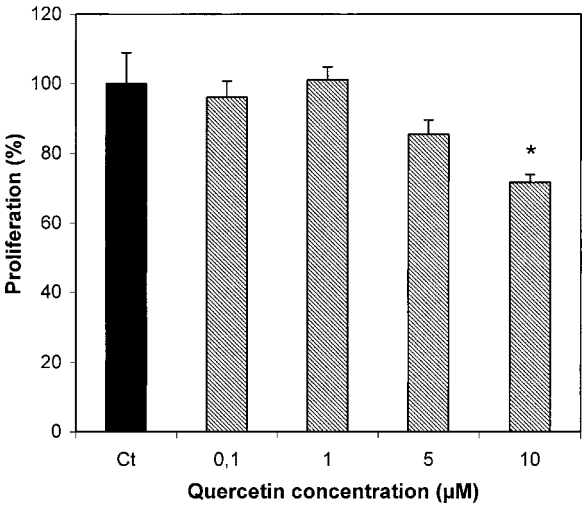

Fig. 4. Evaluation of toxic effect of quercetin on RAW cells using MTT based assay, after 5 days of culture in presence of different concentrations of quercetin. Values are the mean ± SEM of two independent experiments (n = 4 for each experiment). $P < 0.05$ (*) compared with control group.

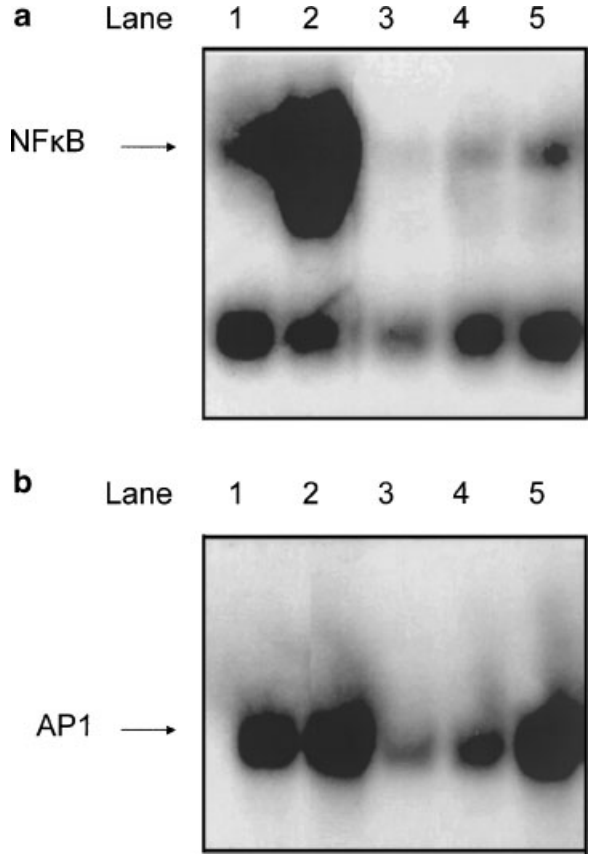

Fig. 5. Effect of quercetin on NFκB (**a**) and AP-1 (**b**) activation in RAW 264.7 cells by electrophoretic mobility shift assay (EMSA). RAW 264.7 were not stimulated (**lane 1**) or stimulated with RANKL (30 ng/ml) for 30 min (**lane 2**). **Lanes 3–5** represent NFκB or AP-1 activation after a 2 h pre-treatment with respective concentrations of quercetin (10, 1, 0.1 μM) followed by a 30 min treatment by RANKL (30 ng/ml).

($r = 0.9$; $P < 0.001$), demonstrating that bone resorption is directly dependent on OCLs number.

OCLs number, TRAP activity, and bone resorption were decreased in a dose-dependent manner in the presence of quercetin and significant results were obtained at concentrations as low as 1 μM. The IC_{50} calculated for each experiment were very similar, between 1 and 1.5 μM. These concentrations are physiologically relevant to the plasmatic concentrations of quercetin reached after consumption of a meal rich in quercetin. Quercetin is regularly consumed by humans and the major sources of quercetin are tea, wine, onions, broccolis, and apples. The average daily consumption of quercetin is about 30 mg per day [de Vries et al., 1997] and depends on dietary habits. Anyway, the daily intake influences the plasmatic concentrations which have been evaluated in micromolar range, even if a weak intestinal absorption has been reported [Hollman et al., 1997]. These plasmatic levels may be sufficient to exert biological effects. However, much higher concentrations should be achieved through the use of pharmaceutical preparations or dietary supplements.

MTT test demonstrated that only the concentration of 10 μM induces a slight decrease in RAW proliferation. Thus, the quercetin-induced inhibitory effect on osteoclastogenesis observed at low concentrations cannot be assimiled to a toxic effect but may involve a modulation of intracellular signaling. We used RAW 264.7 cells to explore the molecular mechanism by which quercetin decreases OCLs formation. We examined its effects on two transcription factors, NFκB and AP-1, which are well known to modulate differentiation, survival, and activation of osteoclasts [Grigoriadis et al., 1994; Jimi et al., 1998]. RANKL has been reported to stimulate NFκB and AP-1 in the processes of osteoclastogenesis [Matsuo et al., 2000; Wei et al., 2001]. Moreover, inhibitory effects of quercetin on NFκB and AP-1 activation have already been reported in other cellular models, especially mesangial cells [Ishikawa et al., 1999; Ishikawa and Kitamura, 2000]. We found that quercetin inhibits RANKL-induced transcription factor activation in osteoclastic precursors. The inhibition of RANKL-induced NFκB and AP-1 activation by quercetin

at low concentrations could be one of the cellular targets involved in the decrease of OCL formation.

The molecular mechanism whereby quercetin can decrease osteoclastogenesis remains to be specified. The best-described property of quercetin is its capacity to act as an antioxidant [Rice-Evans et al., 1995] and we recently demonstrated that quercetin keeps its antioxidant properties in mature osteoclastic cells [Wattel et al., 2003]. Osteoclasts are cells known to produce high amounts of reactive oxygen species (ROS) and these free radicals play an important role not only in bone resorption [Bax et al., 1992], but also in the process of osteoclast differentiation [Suda et al., 1993], possibly involving activation of the transcription factor NFκB and AP-1 [Schreck et al., 1991; Lo et al., 1996]. Additionally, antioxidants like pyrrolidinedithiocarbamate (PDTC) and *N*-acetyl cystein (NAC) can oppose ROS effects by inhibiting NFκB and AP-1 activation [Wolle et al., 1995; Kyaw et al., 2002] and by doing so they can decrease bone resorption [Hall et al., 1995]. Further research is now necessary to precise whether antioxidant capacity of quercetin is involved in its antiosteoclastogenic effect and especially to determine if this property implies mitogen activated protein kinase (MAPK) as extracellular signal regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) that are involved in NFκB and AP-1 activation. Suppression of ERK and p38 activation by quercetin may also lead to a modulation of concentration of nitric oxide (NO), a compound known to be a potent intracellular mediator of bone resorption [van't Hof et al., 2000; Jung et al., 2003].

In conclusion, this study examined the effects of quercetin, a flavonol widely distributed in food of plant origin, on osteoclastogenesis. Using two major models of osteoclast-like cells, we showed for the first time that quercetin inhibits osteoclast differentiation at very low concentrations. Finally, our results showed quercetin negatively regulates NFκB and AP-1 transcriptional activities in osteoclastic precursors. Taken together, these results suggest that quercetin, a natural antioxidant, could be an important nutrient in the regulation of bone resorption.

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