Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 485 (2004) 89–96
www.elsevier.com/locate/ejphar

Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro

Michitaka Notoya[a], Yu Tsukamoto[a], Hiroyuki Nishimura[b], Je-Tae Woo[c], Kazuo Nagai[c], In-Sun Lee[d], Hiromi Hagiwara[a,*]

[a] Department of Biological Sciences, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan
[b] Department of Biomedical Engineering, Toin University of Yokohama, Yokohama 225-8502, Japan
[c] Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan
[d] Center for Traditional Microorganism Resources, Keimyung University, 1000 Shindang-dong, Dasu, Taegu 704-701, South Korea

Received 14 August 2003; received in revised form 14 November 2003; accepted 21 November 2003

Abstract

It is possible that the flavonoids that are found in many foods might have a protective effect against osteoclastic activity. However, little information is available about the effects of flavonoids on osteoblastogenesis. Therefore, we investigated the effects of quercetin, a flavonoid, on the metabolism of rat calvarial osteoblast-like cells (ROB cells) in culture. The proliferation of cells was markedly inhibited upon exposure of cells to quercetin at 5×10^{-6} to 1×10^{-5} M. Quercetin at 1×10^{-5} M did not induce apoptosis in ROB cells but arrested cells at the G1 phase of the cell cycle. In addition, quercetin stimulated the expression of mRNA for $p21^{waf1/cip1}$, which inhibits the activity of cyclin-dependent kinases, and inhibited the phosphorylation of histone H1. Furthermore, after cells had ceased to proliferate, quercetin reduced the activity of alkaline phosphatase, the level of expression of mRNA for osteocalcin, the rate of deposition of Ca^{2+}, and the formation of mineralized nodules, all of which are markers of osteoblast differentiation. These findings indicate that quercetin inhibits the proliferation, differentiation, and mineralization of osteoblastic cells.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Quercetin; Flavonoid; Osteoblast; Proliferation; Differentiation; Cell cycle

1. Introduction

Osteoblasts are bone-forming cells. The formation of bone involves a complex series of events that include the proliferation and differentiation of osteoprogenitor cells and result eventually in the formation of a mineralized extracellular matrix. Numerous cytokines, hormones, and growth factors control the formation of bone by regulating both the proliferation and the differentiation of osteoblasts. Bone metabolic diseases develop when there is an imbalance between the formation and resorption of bone, which, in turn, depend on the interactions between osteoblasts and osteoclasts (Riggs, 1987). In our efforts to identify factors that might cause, prevent, or treat bone metabolic diseases, such as osteoporosis and osteopetrosis, we screened various compounds for the ability to regulate the proliferation, differentiation, and function of cultured osteoblasts and osteoclasts (Hagiwara et al., 1999; Yuhara et al., 1999; Yamagishi et al., 2001).

Quercetin is one of the major flavonoids in certain species of plants (for example, 200–600 mg quercetin/kg onion), in which it is present mainly as glycosides (rutin; Price and Rhodes, 1997). Dietary glycosides are converted to aglycones (such as quercetin) in the large intestine in reactions catalyzed by the glycosidases of intestinal bacteria (Tamura et al., 1999). Rutin inhibits the ovariectomy-stimulated resorption of bone in rats (Horcajada-Molteni et al., 2000), and quercetin has been reported to inhibit the osteoclastic resorption of bone in vitro (Wattel et al., 2003). We also found that quercetin inhibits the formation of osteoclast-like cells that is induced by treatment with the receptor activator of nuclear factor-κB ligand of mouse marrow-derived monocytes whose growth in vitro is dependent on macrophage-colony stimulating factor and the mouse monocyte cell line RAW264.7 (unpublished data). These observations suggest that quercetin might inhibit

* Corresponding author. Tel.: +81-45-924-5720; fax: +81-45-924-5777.
E-mail address: hhagiwar@bio.titech.ac.jp (H. Hagiwara).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.11.058

osteoclastogenesis by affecting osteoclasts. However, the effects of quercetin on the metabolism of osteoblasts remain to be clarified. Therefore, we examined the effects of this flavonoid on osteoblasts, and we found that it decreased the proliferation, differentiation, and mineralization of rat calvarial osteoblast-like cells (ROB cells; Hagiwara et al., 1996; Partridge et al., 1981) in a well-characterized model system that has been used for studies of the proliferation and differentiation of bone-forming cells and of the molecular biology of the mineralization process.

2. Materials and methods

2.1. Materials

Quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one dihydrate] was purchased from Sigma (St. Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO), which was used in control experiments at 0.1%. α-Modified Eagle's Medium (α-MEM), penicillin/streptomycin antibiotic mixture, and fetal bovine serum were obtained from Life Technologies (Grand Island, NY, USA). 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole, trihydrochloride (Hoechst 33342) was purchased from Hoechst (Tokyo, Japan).

2.2. Culture of osteoblastic cells

ROB cells were isolated enzymatically from calvariae of newborn Wistar rats as described previously (Hagiwara et al., 1996). Cells were maintained in 75-cm^2 flasks in α-MEM, supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37 °C. After reaching 70% confluence, cells were detached by treatment with 0.05% trypsin, replated in either 55-cm^2 dishes or 12-well plates (area of each well, 3.8 cm^2) at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. Fresh medium was supplied to cells at 3-day intervals. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

2.3. Quantitation of cell proliferation

ROB cells were replated in 12-well plates (area of each well, 3.8 cm^2) at the low density of 0.25×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and quercetin at various concentrations. Cells were detached by treatment with 0.05% trypsin at the times indicated and were counted under a light microscope (IX70; Olympus, Tokyo, Japan).

2.4. Flow-cytometric analysis

Cells were plated in 55-cm^2 dishes at a density of 0.2×10^4 cells/cm^2 and treated with 1×10^{-5} M quercetin for 48 h. After the exposure of cells to quercetin, cells were harvested in 0.05% trypsin and resuspended in 1 ml of phosphate-buffered saline (pH 7.4; 20 mM sodium phosphate and 130 mM NaCl; PBS) that contained 125 U/ml RNase A (Nacalai tesque, Kyoto, Japan) and 50 μg/ml propidium iodide (Wako, Osaka, Japan). The fluorescence of cells was measured with a FACS Vantage system (Becton Dickinson, San Jose, CA, USA). Data were analyzed with CellFIT software (Becton Dickinson).

2.5. Reverse transcription–polymerase chain reaction

We detected mRNAs for mouse $p21^{waf1/cip1}$ and $p27^{kip1}$ in ROB cells by reverse transcription–polymerase chain reaction (RT-PCR). We extracted RNA from ROB cells using ISOGEN™ (Wako) according to the manufacturer's protocol, and then we reverse transcribed the total RNA (2 μg) using Moloney murine leukemia virus reverse transcriptase, Superscript™ (200 U; Promega, Tokyo, Japan), random primers (50 ng) and a 25-μl reaction mixture. The resultant cDNA was amplified in 20 μl of Taq DNA polymerase mixture (Takara, Tokyo, Japan) that contained 1×10^{-6} M sense primer, 5'-ACGTGGCCTTGTCGCTGTCTT-3', and 1×10^{-6} M antisense primer, 5'-TAAGGCAGAAGATGGGGAAGAG-3', for mouse $p21^{waf1/cip1}$ (accession number V09507; 523–747, 225 bp); 1×10^{-6} M sense primer, 5'-AGGAGAGCTTGGATGTCAG-3', and 1×10^{-6} M antisense primer, 5'-TTTCTTCTGTTCTGTTGGCC-3', for mouse $p27^{kip1}$ (accession number V10440; 528–734, 207 bp); or 1×10^{-6} M sense primer, 5'-ACTTTGTCAAGCTCATTTCC-3', and 1×10^{-6} M antisense primer, 5'-TGCAGCGAACTTTATTGATG-3', for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number M32599; 957–1223, 267 bp). Each amplification cycle, repeated a total of 35, 40, and 30 times for $p21^{waf1/cip1}$, $p27^{kip1}$, and GAPDH, respectively, consisted of incubation at 94 °C for 30 s, at 60 °C for 30 s, and 72 °C for 45 s. Products of PCR were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA marker fragments (Molecular weight marker V; Boehringer Mannheim, Mannheim, Germany) were used as size markers.

2.6. In vitro histone H1 kinase assay

ROB cells were plated in 55-cm^2 dishes at a density of 0.2×10^4 cells/cm^2 and treated with 1×10^{-5} M quercetin for 48 h. Histone H1 kinase activity was assayed in cyclin-cdk complexes isolated from cells treated with quercetin or DMSO for 48 h. Cells were mixed with four volume of ice-cold extraction buffer [160 mM sodium-β-glycerophos-

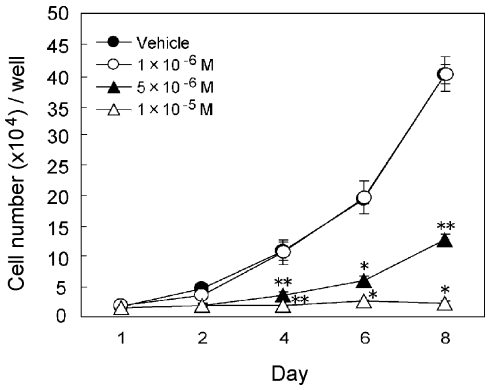

Fig. 1. Effects of quercetin on the proliferation of osteoblasts. ROB cells were plated in 12-well plates (3.8 cm^2/well) at the low density of 0.25×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum and quercetin at various concentrations as indicated. Fresh medium with quercetin was supplied at 3-day intervals. At the times indicated, cells were removed from plates with a 0.05% solution of trypsin and counted under a light microscope. *$P < 0.002$ vs. vehicle; **$P < 0.01$ vs. vehicle.

phate, 40 mM EGTA, 30 mM $MgCl_2$, 200 mM KCl, 200 mM sucrose, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 0.1% Nonidet P-40 (polyoxyethylene(9)octylphenyl ether), 50 mM benzamidine, 50 mM phenylmethanesulfonyl fluoride, 50 mg/ml leupeptin, and 50 mg/ml soybean trypsin inhibitor, pH 7.3], followed by immediate freezing in liquid nitrogen. After thawing, the cell suspension was vortexed five times for 10 s on ice. After centrifugation at 12,000 × *g* for 10 min at 4 °C, the supernatant was recovered to a fresh tube as cell extract.

To isolate cyclin-cdk complexes, 10 μl of cell extracts were added to 5 μl Suc1-beads and incubated for 1 h at 4 °C. The complexes were washed five times with 1 ml of kinase buffer (80 mM sodium-β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.3), resuspended in 5 μl of histone H1-ATP mixture (1.3 mg/ml histone H1, 0.65 mM cold ATP, and 0.22 mCi/ml [γ-^{32}P]ATP), and then incubated for 30 min at 25 °C. The reactions were stopped by the addition of 10 μl of sample buffer. Samples were run on sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis, and the gel was exposed to an imaging plate for 10 min. The signals on the plate were analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan). The radioactivity of the excised histone H1 bands was quantified by using a liquid scintillation counter.

2.7. Measurement of alkaline phosphatase activity

ROB cells were subcultured in 12-well plates (3.8 cm^2/well) in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence (day 3), quercetin was added at various concentrations to the culture medium. Fresh medium containing quercetin was supplied at 3-day intervals. At indicated times, cells were washed with 10 mM Tris–HCl, pH 7.2, and sonicated for 15 s in 1 ml of 50 mM Tris–HCl, pH 7.2, that contained 0.1% Triton X-100 and 2 mM $MgCl_2$ in a sonicator (Ultrasonic Disruptor UD-201; Tomy Tokyo, Japan). The alkaline phosphatase activity of each sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate. Concentrations of protein were determined with bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as the standard.

2.8. Northern blotting analysis

Total RNA was isolated from ROB cells that had been treated with quercetin or vehicle for indicated times. Then, total RNA (10 μg) was subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde and the bands of RNA were transferred to a nylon membrane (Hybond-N+; Amersham Bioscience, Uppsala, Sweden). After the membrane had been baked at 80 °C for 2 h, the RNA on the membrane was allowed to hybridize overnight with cDNAs for rat osteocalcin and GAPDH at 42 °C in PerfectHyb® Hybridization Solution (TOYOBO, Tokyo, Japan). Each cDNA probe was radiolabeled with a Ready-to-Go™ kit (Amersham Bioscience). The membrane was washed twice for 5 min each in 1× sodium chloride/sodium citrate (SSC; 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature and twice for 1 h each in 1 × SSC that contained 0.1% SDS at 55 °C, and then, it was exposed to an imaging plate overnight. The signals on the plate were analyzed with a Bioimage Analyzer.

2.9. Quantitation of the deposition of Ca^{2+}

ROB cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence (day 3), quercetin was added at various concentrations to the culture medium. The amount of Ca^{2+} deposited in the cell layer was measured as follows. The layers of cells in 12-

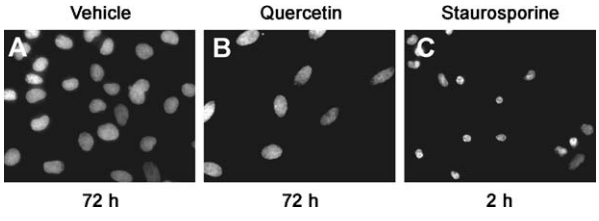

Fig. 2. The apoptotic effects of quercetin on ROB cells. ROB cells were stained with Hoechst 33342 for examination of nuclei. The cells were incubated with and without 1×10^{-5} M quercetin for 3 days and, as a positive control, with 1×10^{-5} M staurosporine for 2 h, and then they were treated with 0.2 μg/ml Hoechst 33342 for 15 min at 37 °C. Photographs show stained cells that had been treated as follows: (A) with vehicle (0.1% DMSO) for 3 days; (B) with 1×10^{-5} M quercetin for 3 days; and (C) with 1×10^{-5} M staurosporine for 2 h. Bar = 10 μm.

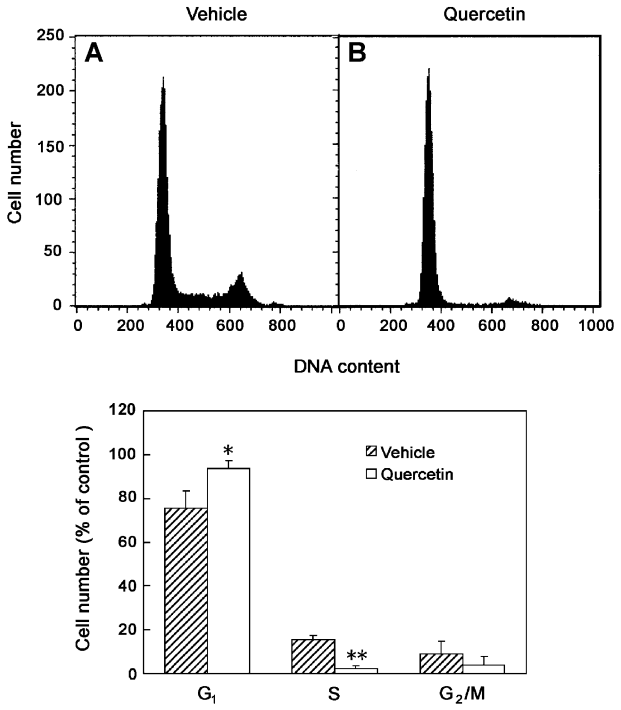

Fig. 3. Flow-cytometric analysis of proliferating cultures of ROB cells that had been cultured in the absence (A) and in the presence (B) of 1×10^{-5} M quercetin for 48 h. The fluorescence of the cells was monitored with a FACS Vantage system, and data were analyzed with CellFIT software as described in the text. The cell cycle was analyzed by RFIT model analysis, and the results are shown as a histogram, $^{*}P<0.05$ versus vehicle, $^{**}P<0.01$ versus vehicle.

well plates (3.8 cm^2/well) were washed with PBS and incubated overnight with 1 ml of 2N HCl with gentle shaking. The Ca^{2+} ions in the samples were quantitated by the *o*-cresolphthalein complexone method with a Calcium C kit (Wako). This kit is specific for Ca^{2+} ions and has a limit of detection of 1 μg/ml. We used the solution of Ca^{2+} ions (20 mg/dl) in the kit as the standard solution.

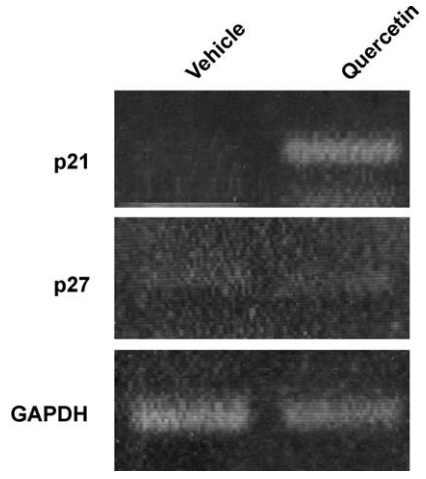

Fig. 4. Expression of mRNA for $p21^{waf1/cip1}$ in ROB cells in the presence and absence of 1×10^{-5} M quercetin. Total RNA was isolated from ROB cells and levels of mRNAs for mouse $p21^{waf1/cip1}$ (p21; 225 bp), $p27^{kip1}$ (p27; 207 bp), and mouse GAPDH (267 bp) were examined by RT-PCR with specific primers (see text for details).

2.10. von Kossa staining

ROB cells that had been cultured with and without 1×10^{-5} M quercetin for 12 days in 12-well plates (3.8 cm^2/well) were fixed in 10% formaldehyde for 30 min at room temperature and then washed three times with 10 mM Tris–HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in sunlight, washed twice with H_2O, and then treated with 5% sodium thiosulfate.

2.11. Statistical analysis

Numerical data were expressed as means ± S.D. of results from three or four cultures, and the significance of differences was analyzed by Student's *t*-test. Statistical

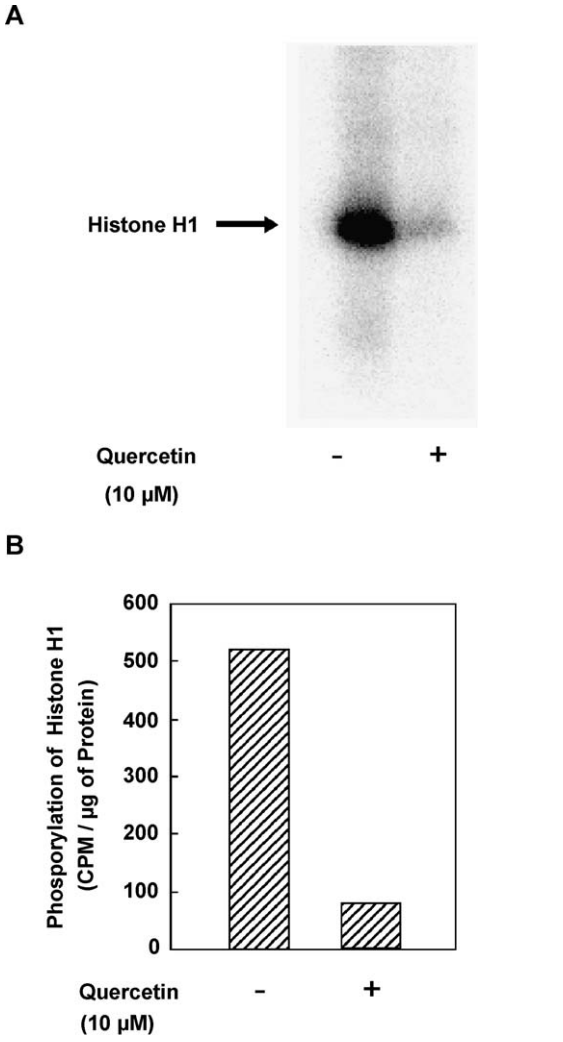

Fig. 5. Effects of quercetin on the histone H1 kinase activity in ROB cells. (A) In vitro histone H1 kinase activity of ROB cells treated with 1×10^{-5} M quercetin for 48 h. (B) The radioactivity of the histone H1 band of panel (A).

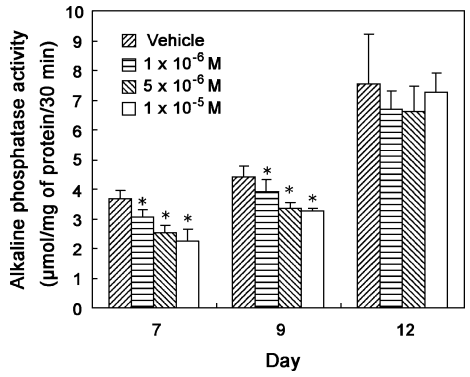

Fig. 6. Effects of quercetin on the alkaline phosphatase activity of ROB cells. ROB cells in 12-well plates (3.8 cm^2/well) were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence (day 3), quercetin was added at various concentrations to the culture medium as indicated. Fresh medium with quercetin was supplied at 3-day intervals. At the times indicated, alkaline phosphatase activity was measured as described in the text. *$P<0.05$ vs. vehicle.

significance was recognized at $P<0.05$. Experiments were repeated independently in triplicate and the results were qualitatively identical in every case. Results from representative experiments are shown.

3. Results

3.1. Effects of quercetin on cell proliferation

The exposure of ROB cells to 5×10^{-6} M quercetin decreased the number of cells by approximately 70% in 8 days, as compared with control cultures that were treated with vehicle alone and the effects of quercetin were dose dependent (Fig. 1). Phase contrast microscopy revealed that ROB cells became slightly hypertrophic during exposure to 1×10^{-5} M quercetin. We confirmed, using Hoechst 33342, that the effects of quercetin were not attributable to the induction of apoptosis in ROB cells under our cell-culture conditions. As shown in Fig. 2, the nuclei of ROB cells that had been treated with 1×10^{-5} M quercetin for 72 h were similar to those of control cells. By contrast, cells treated with 1×10^{-5} M staurosporine for 2 h had condensed nuclei (positive control).

We examined the effects of quercetin on progression of the cell cycle and found that incubation of ROB cells with 1×10^{-5} M quercetin increased the number of ROB cells in the G1 phase of the cell cycle (Fig. 3). The percentages of ROB cells that had been treated with 1×10^{-5} M quercetin for 48 h in the G1, S, and G2/M phases were 93.7%, 2.5%, and 3.8%, respectively. By contrast, the values for control cells were 75.7%, 15.4%, and 9.0%, respectively. Next, to determine the mechanism responsible for the arrest of the cell cycle by quercetin, we examined the levels of expression of mRNAs for the inhibitors of cyclin-dependent kinases (cdks) $p21^{waf1/cip1}$ and $p27^{kip1}$ in quercetin-treated ROB cells. As shown in Fig. 4, quercetin stimulated the expression of mRNA for $p21^{waf1/cip1}$. We were unable to detect the expression of the mRNA for $p27^{kip1}$ in ROB cells. Furthermore, 1×10^{-5} M quercetin markedly inhibited the phosphorylation of histone H1 (Fig. 5).

3.2. Effects of quercetin on the differentiation of osteoblastic cells

We examined the effects of quercetin on the osteoblastic differentiation of ROB cells after they had proliferated by exposing confluent ROB cells to quercetin at various concentrations. Fig. 6 showed that quercetin decreased the alkaline phosphatase activity of ROB cells in a dose-dependent manner. The alkaline phosphatase activity of ROB cells that had been exposed to 1×10^{-6} M quercetin for 7 and 9 days was significantly lower than that in control cells (treated with DMSO). On day 12, we were unable to

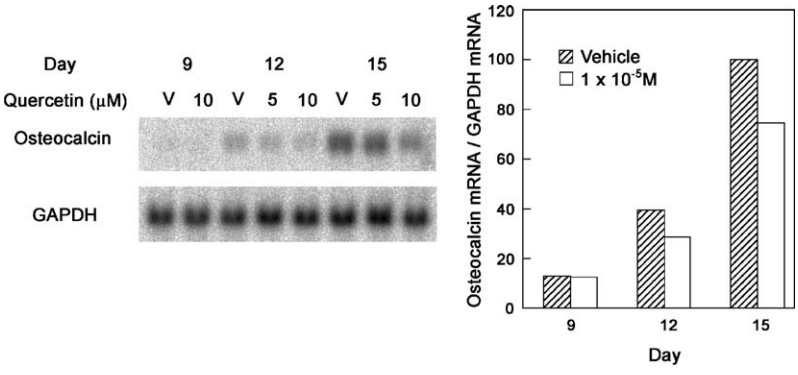

Fig. 7. Northern blotting analysis of mRNA for osteocalcin (OC) in ROB cells. ROB cells in 55-cm^2 dishes were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence (day 3), quercetin was added to the culture medium as indicated. On days 9, 12, and 15, total RNA was isolated from cells, and 20 μg of total RNA was fractionated by electrophoresis on a 1% agarose gel. Then, bands of RNA were allowed to hybridize with ^{32}P-labeled cDNAs for rat osteocalcin and GAPDH (V, results for cells treated with 0.1% DMSO). The relative level of osteocalcin mRNA was determined with a Bioimage Analyzer, with normalization by reference to the level of the mRNA for GAPDH. The ratio of the level of osteocalcin mRNA to the level of GAPDH mRNA on day 15 for cells treated with vehicle was defined as 100%.

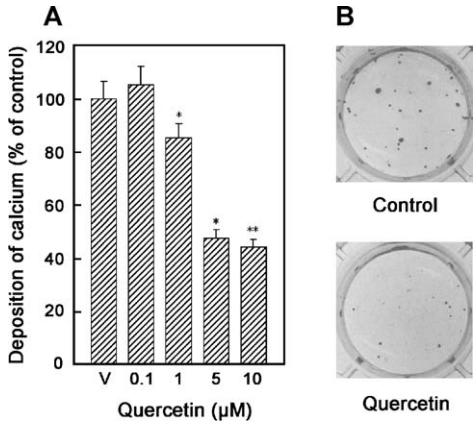

Fig. 8. Effects of quercetin on the mineralization of osteoblastic cells. ROB cells in 12-well plates (3.8 cm^2/well) were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence (day 3), quercetin was added at various concentrations to the culture medium as indicated. Fresh medium with quercetin was supplied at 3-day intervals. (A) The deposition of Ca^{2+} ions was measured on day 12. Quantitative analysis of Ca^{2+} ions was performed as described in Materials and methods. *$P<0.05$ vs. vehicle (V); **$P<0.01$ vs. vehicle (V). (B) Phase-contrast photomicrographs. ROB cells were cultured with and without 1×10^{-5} M quercetin for 12 days and subjected to von Kossa staining for visualization of mineralized nodules.

detect any significant inhibitory effect of quercetin on alkaline phosphatase activity in ROB cells. Northern blotting analysis revealed that quercetin inhibited the expression of the mRNA for osteocalcin, which is a marker of osteoblastic differentiation (Fig. 7). On day 12, we detected osteocalcin mRNA in control cells, and the level of expression of this mRNA continued to increase with time. However, the level of expression of the mRNA for osteocalcin in ROB cells that had been treated with 5×10^{-6} and 1×10^{-5} M quercetin was clearly lower than it was in control cells after 15 days. Fig. 8A demonstrates that quercetin decreased the deposition of Ca^{2+} by ROB cells when deposition was examined on day 12. Fig. 8B shows mineralized nodules, after staining with von Kossa's reagents, in cultures of ROB cells on day 12. The number of mineralized nodules was clearly lower in ROB cells that had been treated for 12 days with 5×10^{-6} M quercetin.

4. Discussion

The aim of this study was to clarify the effects of quercetin on the metabolism of osteoblasts. Using rat calvarial primary osteoblast-like cells, ROB cells, that had previously been well characterized, we found that quercetin inhibited not only the proliferation but also the differentiation and mineralization of osteoblasts.

Our results show that quercetin inhibits the proliferation of ROB cells. Quercetin induces apoptosis in a variety of tumor cells, such as Burkitt's lymphoma and human ovarian cancer cells (MCAS; Wei et al., 1994), colorectal tumor cells (Richter et al., 1999), human breast cancer cells (Choi et al., 2001), and murine melanoma B16-BL6 cells (Zhang et al., 2000). However, we failed to detect the induction of apoptosis by quercetin in ROB cells under our culture conditions. By contrast, we found that quercetin caused the accumulation of ROB cells at the G1 phase of the cell cycle specifically, and we detected an increase in the expression of $p21^{WAF1/CIP1}$ mRNA, an inhibitor of cdks. It has been reported that quercetin causes the arrest of the cell cycle (Choi et al., 2001; Zhang et al., 2000) and induces the enhanced expression of $p21^{WAF1/CIP1}$ (Choi et al., 2001; Zhang et al., 2000; Iwao and Tsukamoto, 1999). It has also been reported that $p21^{WAF1/CIP1}$ mediates G1 arrest (Deng et al., 1995). Furthermore, we found that quercetin treatment decreased the phosphorylation of histone H1 in ROB cells. Thus, the inhibitory effect of quercetin on the proliferation of ROB cells might be due, in part, to the arrest of the cell cycle via expression of $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ has recently been reported to act as a brake in osteoblast differentiation (Bellosta et al., 2003).

After ROB cells had reached confluence, quercetin inhibited both differentiation and mineralization, as indicated by markers that are characteristic of the osteoblast phenotype, such as alkaline phosphatase activity, the synthesis of osteocalcin, and the deposition of Ca^{2+}. The mechanism of the quercetin-induced effects on the differentiation of osteoblasts is unknown. Quercetin has been reported to reduce the basal levels of receptors for insulin-like growth factor-I (IGF-I) and of phosphatidylinositol 3-kinase in keloid fibroblasts (Phan et al., 2003). Quercetin is also an inhibitor of phosphatidylinositol 3-kinase (Brown et al., 1995). IGF-I and its signaling pathways play important roles in osteoblastic differentiation (Thomas et al., 1999; Zhao et al., 2000). Therefore, quercetin might influence osteoblastic differentiation via suppression of the signaling pathway(s) of IGF-I in ROB cells. Alternatively, quercetin might act by inhibiting the activity of activator protein-1 (AP-1). AP-1 is a dimeric transcription factor that consists of a Fos-related protein (c-Fos, FosB, Fra-1, Fra-2, or ΔFosB) and a Jun-related protein (JunB, c-Jun, or JunD). Increased formation of bone has been observed in mice that overexpress the transcription factors Fra-1 (Jochum et al., 2000) and ΔFosB (Sabatakos et al., 2000). However, quercetin has also been reported to limit the extent of activation of AP-1 (Ishikawa et al., 1999, 2000).

Quercetin had significant effects on the metabolism of cultured osteoblasts in the present study. The concentrations (1×10^{-6} to 1×10^{-5} M) of quercetin that we used in this study were in the range of those (1×10^{-6} to 1×10^{-4} M) used in studies of the inhibition of the formation and activity of osteoclasts (Wattel et al., 2003). Furthermore, similar concentrations of quercetin have been used in studies of apoptosis (Richter et al., 1999; Ishikawa et al., 1999). Quercetin is one of the most abundant flavonoids in the human diet. Measured concentrations of quercetin glycoside in human plasma ranged from 5×10^{-7} to 1.6×10^{-6} M

(Paganga and Rice-Evans, 1997), and they correspond closely to concentrations that inhibited the proliferation and differentiation of osteoblasts in this study. Thus, our study revealed the similarity in terms of effective inhibitory doses of quercetin between osteoblast and osteoclast metabolism and the effects of high but physiological concentrations of quercetin on cultured osteoblasts.

We have screened isoflavones for the ability to regulate the proliferation, differentiation, and function of cultured osteoblasts and osteoclasts. To date, we reported that synthetic flavonoid ipriflavone increased the deposition of Ca^{2+} by ROB cells (Hagiwara et al., 1999). Furthermore, isoflavone genistein decreased the level of receptor activator of nuclear factor-κB ligand mRNA and increased the level of osteoprotegerin mRNA in osteogenic/stromal cells (Yamagishi et al., 2001). Recently, genistein has been reported to increase the expression levels of osteoprotegerin by osteoblastic MC3T3-E1 cells via an estrogen-receptor-dependent pathway (Chen et al., 2003). Quercetin also acts on osteoclasts to inhibit osteoclastogenesis (Wattel et al., 2003). Our present results show that quercetin inhibits osteoblast metabolism. These observations together suggest that quercetin might be an inhibitor of the metabolism of both cultured osteoblasts and osteoclasts. Rutin has been reported to inhibit the ovariectomy-stimulated resorption of bone in rats (Horcajada-Molteni et al., 2000). However, it is unclear whether quercetin increases or decreases bone mass in vivo. Further research is needed to elucidate the contribution of quercetin to bone metabolism in vivo.

Acknowledgements

The authors thank Dr. K. Ohsumi, Dr. E. Okumura, and Mr. R. Matsuoka for histone H1 kinase assay, and Mrs. R. Arai for her skilled technical support. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

References

Bellosta, P., Masramon, L., Mansukhani, A., Basilico, C., 2003. $p21^{WAF1/CIP1}$ acts as a brake in osteoblast differentiation. J. Bone Miner. Res. 18, 818–826.

Brown, W.J., DeWald, D.B., Emr, S.D., Plutner, H., Balch, W.E., 1995. Role for phosphatidylinositol-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. J. Cell Biol. 130, 781–796.

Chen, X., Garner, S.C., Quarles, L.D., Anderson, J.J.B., 2003. Effects of genistein on expression of bone markers during MC3T3-E1 osteoblastic cell differentiation. J. Nutr. Biochem. 14, 342–349.

Choi, J.A., Kim, J.Y., Lee, J.Y., Kang, C.M., Kwon, H.J., Yoo, Y.D., Kim, T.W., Lee, Y.S., Lee, S.J., 2001. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int. J. Oncol. 19, 837–844.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., Leder, P., 1995. Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. Cell 82, 675–684.

Hagiwara, H., Inoue, A., Yamaguchi, A., Yokose, S., Furuya, M., Tanaka, S., Hirose, S., 1996. cGMP produced in response to ANP and CNP regulates proliferation and differentiation of osteoblastic cells. Am. J. Physiol. 270, C1311–C1318.

Hagiwara, H., Naruse, M., Adachi, C., Inoue, A., Hiruma, Y., Otsuka, E., Naruse, K., Demura, H., Hirose, S., 1999. Ipriflavone down-regulates the expression of endothelin receptors during the differentiation of rat calvarial osteoblast-like cells. J. Biochem. 126, 168–173.

Horcajada-Molteni, M.-N., Crespy, V., Coxam, V., Davicco, M.-J., Remesy, C., Barlet, J.-P., 2000. Rutin inhibits ovariectomy-induced osteopenia in rats. J. Bone Miner. Res. 15, 2251–2258.

Ishikawa, Y., Kitamura, M., 2000. Anti-apoptotic effect of quercetin: intervention in the JNK- and ERK-mediated apoptotic pathways. Kidney Int. 58, 1078–1087.

Ishikawa, Y., Sugiyama, H., Stylianou, E., Kitamura, M., 1999. Bioflavonoid quercetin inhibits interleukin-1-induced transcriptional expression of monocyte chemoattractant protein-1 in glomerular cells via suppression of nuclear factor-κB. J. Am. Soc. Nephrol. 10, 2290–2296.

Iwao, K., Tsukamoto, I., 1999. Quercetin inhibited DNA synthesis and induced apoptosis associated with increase in c-fos mRNA level and the upregulation of p21WAF1CIP1 mRNA and protein expression during liver regeneration after partial hepatectomy. Biochim. Biophys. Acta 1427, 112–120.

Jochum, W., David, J.P., Elliott, C., Wutz, A., Plenk Jr., H., Matsuo, K., Wagner, E.F., 2000. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. Nat. Med. 6, 980–984.

Paganga, G., Rice-Evans, C.A., 1997. The identification of flavonoids as glycosides in human plasma. FEBS Lett. 401, 78–82.

Partridge, N.C., Alcorn, D., Michelangeli, V.P., Kemp, B.E., Ryan, G.B., Martin, T.J., 1981. Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. Endocrinology 108, 213–219.

Phan, T.T., See, P., Tran, E., Nguyen, T.T.T., Chan, S.Y., Lee, S.T., Huynh, H., 2003. Suppression of insulin-like growth factor signaling pathway and collagen expression in keloid-derived fibroblasts by quercetin: its therapeutic potential use in the treatment and/or prevention of keloids. Br. J. Dermatol. 148, 544–552.

Price, K.R., Rhodes, M.J.C., 1997. Analysis of the major flavonol glycosides present in four varieties of onion (*Allium cepa*) and changes in composition resulting from autolysis. J. Sci. Food Agric. 74, 331–339.

Richter, M., Ebermann, R., Marian, B., 1999. Quercetin-induced apoptosis in colorectal tumor cells: possible role of EGF receptor signaling. Nutr. Cancer 34, 88–99.

Riggs, B.L., 1987. Pathogenesis of osteoporosis. Am. J. Obstet. Gynecol. 156, 1342–1346.

Sabatakos, G., Sims, N.A., Chen, J., Aoki, K., Kelz, M.B., Amling, M., Bouali, Y., Mukhopadhyay, K., Ford, K., Nestler, E.J., Baron, R., 2000. Overexpression of ΔFosB transcription factor(s) increases bone formation and inhibits adipogenesis. Nat. Med. 6, 985–990.

Tamura, G., Gold, C., Fezz-Luzi, A., Ames, B.N., 1999. Fecalase: a model for activation of dietary glycosides to mutagens by intestinal flora. Proc. Natl. Acad. Sci. U. S. A. 77, 4961–4965.

Thomas, T., Gori, F., Spelsberg, T.C., Khosla, S., Riggs, B.L., Conover, C.A., 1999. Response of bipotential human marrow stromal cells to insulin-like growth factors: effect on binding protein production, proliferation, and commitment to osteoblasts and adipocytes. Endocrinology 140, 5036–5044.

Wattel, A., Kamel, S., Mentaverri, R., Lorget, F., Prouillet, C., Petit, J.-P., Fardelonne, P., Brazier, M., 2003. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochem. Pharmacol. 65, 35–42.

Wei, Y.Q., Zhao, X., Kariya, Y., Fukata, H., Teshigawara, K., Uchida, A., 1994. Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res. 15, 4952–4957.

Yamagishi, T., Otsuka, E., Hagiwaram, H., 2001. Reciprocal control of expression of mRNAs for osteoclast differentiation factor and OPG in osteogenic stromal cells by genistein: evidence for the involvement of topoisomerase II in osteoclastogenesis. Endocrinology 142, 3632–3637.

Yuhara, S., Kasagi, S., Inoue, A., Otsuka, E., Hirose, S., Hagiwara, H., 1999. Effects of nicotine on cultured cells suggest that it can influence the formation and resorption of bone. Eur. J. Pharmacol. 383, 387–393.

Zhang, X., Xu, Q., Saiki, I., 2000. Quercetin inhibits the invasion and mobility of murine melanoma B16-BL6 cells through inducing apoptosis via decreasing Bcl-2 expression. Clin. Exp. Metastasis 18, 415–421.

Zhao, G., Monier-Faugere, M.C., Langub, M.C., Geng, Z., Nakayama, T., Pike, J.W., Chernausek, S.D., Rosen, C.J., Donahue, L.R., Malluche, H.H., Fagin, J.A., Clemens, T.L., 2000. Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. Endocrinology 141, 2674–2682.