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Effects of Quercetin on TNF-α-Induced Cytokine Secretion and Nitric Oxide Production in MC3T3-E1 Osteoblastic Cells

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Abstract – Bioflavone quercetin is thought to have an important role to inhibit bone loss by affecting osteoclastogenesis and regulating a number of systemic and local factors such as hormones and cytokines. In this study, we examined how quercetin acts on cytokine production and mineralization of osteoblast in the presence of tumor necrosis factor-alpha (TNF-α) which has been known to play a pivotal role in bone metabolic diseases. Quercetin inhibited TNF-α-induced secretion of IFN-γ and IL-6 in differentiated MC3T3-E1 cells. As indicated by the markers that are characteristics of the osteoblast phenotype, such as alkaline phosphatase (ALP) activity and calcium deposition, quercetin treatment slightly prevented the TNF-α-induced dramatic inhibition of differentiation and mineralization of MC3T3-E1 cells. Further, quercetin inhibited the production of nitric oxide induced by TNF-α in the cells. Collectively, our findings indicate that quercetin inhibites TNF-α-induced secretion of inflammatory cytokines in differentiated MC3T3-E1 cells without any cytotoxic effects.
Keywords – quercetin, TNF-α, MC3T3-E1 osteoblastic cells, inflammatory cytokine

Introduction

Bone is continuously remodeled in order to maintain bone mass and calcium homeostasis through a balanced interaction between osteoblasts and osteoclasts which are specialized cells responsible for bone formation and resorption, respectively (Katagiri and Takahashi, 2002). Bone cells and their interactions are very sensitive to systemic and local factors such as hormones and cytokines. Among the various hormones and cytokines involved in bone metabolic regulation, tumor necrosis factor-alpha (TNF-α) has been known to play a pivotal role in osteoporosis. TNF-α stimulates osteoblasts to secrete other inflammatory cytokines such as interleukin (IL)-1 B and IL-6, and prostaglandin E_2 (PGE_2) as well as TNF-α itself, which directly act on osteoclasts to cause bone resorption (Franchimont *et al.*, 1997; Jilka, 1998; Glantschnig *et al.*, 2003). In addition, TNF-α induces apoptosis of osteoblasts (Chua *et al.*, 2002; Suh *et al.*, 2003). Thus, it is believed that an increase of TNF-α level and a decrease in osteoblast cell numbers via apoptosis could be responsible for the bone loss, and if these conditions persist, osteoporosis occurs.

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In recent years, there has been a global trend toward the use of natural bioactive compounds as chemoregulators of the bone cells (Choi and Koo, 2003; Suh *et al.*, 2003; Gallagher *et al.*, 2004; Kreijkamp-Kaspers *et al.*, 2004). Isoflavones such as daidzein and genistein, which are found in abundance in soybeans, have been shown reducing the occurrence of osteoporosis in various experiments (Arjmandi *et al.*, 1996; Morabito *et al.*, 2002). In addition, a dietary uptake of flavonoid rich plants is closely associated with the low incidence of osteoporosis (Cassidy, 2003). Quercetin (3,3',4',5,7-pentahydroxyflavone), a dietary flavone that is commonly found in plants, has been reported to have biological, pharmacological, and medicinal activities (Morel *et al.*, 1993; Hollman and Katan, 1999). In recent, it has been reported that quercetin inhibits the osteoclastic resorption of bone *in vitro* (Wattel *et al.*, 2003). It also found that quercetin has suppressive effect on bone resorption by inhibiting the differentiation of osteoclast progenitor cells into preosteoclasts and by disrupting the formation of actin rings in mature osteoclasts (Woo *et al.*, 2004). These investigations suggest that quercetin plays an important role in inhibiting bone loss by affecting osteoclastogenesis and regulating a number of systemic and local factors such as hormones and inflammatory cytokines. However, the precise effects

of quercetin on the differentiation and function of osteoblasts remain to be further clarified. Therefore, we investigated the effects of quercetin on cytokine production and mineralization of osteoblasts in the presence of TNF-α.

Experimental

Chemicals and laboratory wares – Unless otherwise specified, chemicals and laboratory ware were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. Quercetin (Q0125; Sigma Chemical Co.) was dissolved in ethanol immediately before use and the final concentration of ethanol did not exceed 0.1% (v/v) throughout the experiments.

Cell culture and treatment – The murine osteoblastic MC3T3-E1 cells (ATCC, CRL-2593) were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics. The cultures were maintained at 37°C with a gas mixture of 5% CO_2/95% air and subcultures were performed with 0.05% trypsin-0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (DPBS; Gibco BRL Co., USA). MC3T3-E1 cells (1×10^5 cells/ml) were replated in the 24-well flat-bottomed plates and cultured in the α-MEM supplemented with 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. The fresh medium was supplied to cells at 3-day intervals. After reaching almost complete confluence, the cells were exposed to TNF-α in the presence and absence of quercetin. At various times after the treatment, the cells were processed for the analyses of cytokine production, nitric oxide production and mineralization.

Measurement of cytokine levels – The amount of cytokines produced by the quercetin and/or TNF-α-stimulated MC3T3-E1 cells was determined by ELISA which was provided by the Bank for Cytokine Research (Chonbuk National University, Chonju, Korea) as described previously (VanCott *et al.*, 1996). Briefly, MC3T3-E1 cells cultured in 24-well tissue culture plates were treated with TNF-α and/or quercetin for 72 h and then the culture supernatants were collected. Levels of cytokines such as IFN-γ, IL-6, and IL-1β were determined by ELISA. The amounts of cytokines produced were calculated from standard curves generated using known concentrations of recombinant cytokine proteins.

Measurement of alkaline phosphatase (ALP) activity – MC3T3-E1 cells were subcultured in 24-well flatted bottom plates in α-MEM supplemented with 10% FBS, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. After cells had reached confluence, various concentrations of quercetin without or with 10 ng/ml TNF-α were added to the culture medium. At various times of the treatment, the cells were collected and vigorously resuspended in a lysis buffer (50 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, and 2 mM $MgCl_2$). ALP activity of each sample was determined by an established technique with *para*-nitrophenyl phosphate (pNPP) as the substrate. Briefly, 200 μl of ALP yellow (pNPP) liquid substrate (Sigma Chemical Co., A3469) for ELISA was transferred to each well of 96-multiwell ELISA plates, and then each sample (50 μl) was added to the wells. After incubation, the reaction was stopped by addition of 50 μl of 3 N NaOH and optical density was measured at 405 nm using a SpectraCount™ (Packard Instrument Co., Downers Grove, IL, USA) ELISA reader.

Determination of Ca^{2+} deposition – After MC3T3-E1 cells had reached confluence in α-MEM supplemented 10% FBS, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, quercetin (1-10 μM) and/or TNF-α (10 ng/ml) were added to the culture medium. At various times (1-3 weeks) of the treatment, the amounts of Ca^{2+} deposited in cell layer were measured by *o*-cresolphthalein complexone method with a Calcium C kit (Wako Chemical Co., Osaka, Japan).

Measurement of nitric oxide – MC3T3-E1 cells were treated with quercetin in the absence or presence of 10 ng/ml TNF-α for various times and then culture supernatants were collected. Nitric oxide production was measured as a function of nitrite (NO_2^-) concentration by the method of Green *et al.* (1982). Briefly, the supernatants (50 μl) were mixed with 100 μl of 1% sulfanilamide and 100 μl of 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid and incubated for 5 min at room temperature. Absorbance was measured at 540 nm using a SpectraCount™ ELISA reader, and $NaNO_2$ was used as a standard for the quantification of NO_2^-.

Determination of antioxidant property of quercetin – TNF-α-induced production of reactive oxygen species (ROS) in MC3T3-E1 cells was detected using flow cytometric analysis as described by Bass *et al.* (1983). Briefly, a stock solution of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (50 mM; Calbiochem, Darmstadt, Germany) was prepared in dimethyl sulfoxide (DMSO) and stored at –20°C in the dark. MC3T3-E1 cells were incubated for 24 h with 10 ng/ml TNF-α without or with quercetin pre-incubation, and subsequently incubated with 25 μM DCFH-DA for 20 min. The green fluorescence of DCF was recorded at 515 nm (FL 1) using a FACS calibur® system (Becton Dickinson, San Jose, CA, USA) and 10,000 events were counted per sample.

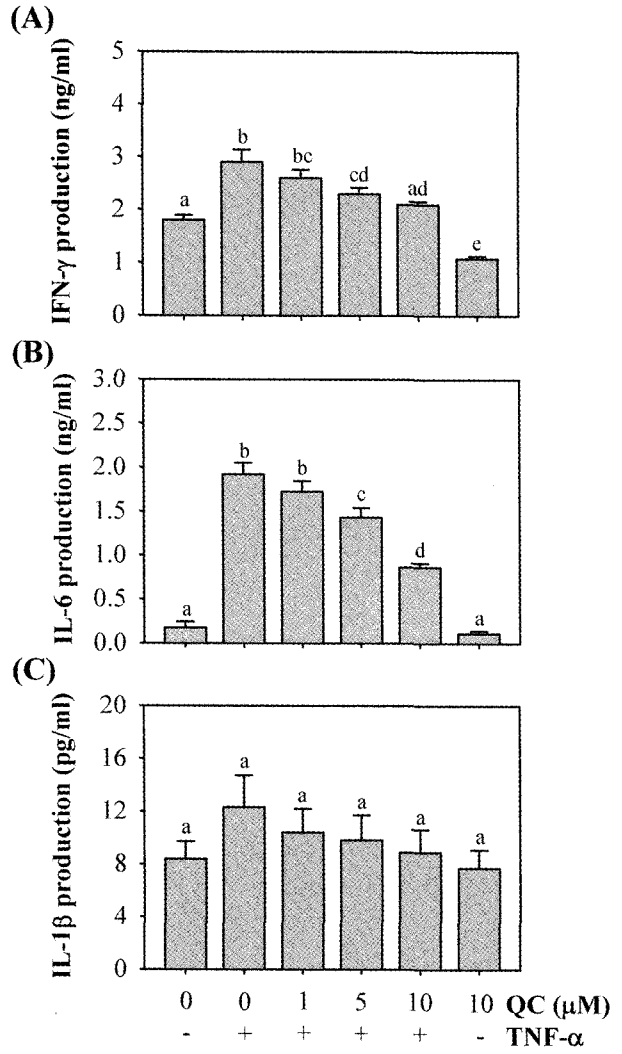

Fig. 1. Inhibitory effects of quercetin on TNF-α-mediated cytokine secretion by MC3T3-E1 cells.

MC3T3-E1 cells were exposed to 10 ng/ml TNF-α in the presence of 1 to 10 μM quercetin for 72 h and the levels of IFN-γ (A), IL-6 (B), and IL-1β (C) were analyzed by ELISA. The same experiments were repeated three times and the figure is a representative result expressed as the mean ± SE. Different superscripts represent significant differences ($P < 0.05$) among groups by Duncan's multiple range test. QC, quercetin.

Statistical analyses – The results are expressed as mean ± standard error (SE). Statistical analysis was performed using ANOVA (SPSS ver. 10.0 software) followed by Duncan's test and a value of $P < 0.05$ was considered significant.

Results and Discussion

TNF-α stimulates osteoblasts to secrete inflammatory cytokines (Franchimont *et al.*, 1997; Jilka, 1998). It is hypothesized that a blockage of the inflammatory cytokines may induce osteoblastic activation, while it suppresses osteoclastic activation. Indeed, a lot of dietary polyphenols inhibited the secretion of inflammatory cytokines in bone cells (Choi and Hwang, 2003; Choi and Koo, 2003; Suh *et al.*, 2003).

As an initial attempt to understand the acting mechanism(s) of quercetin on osteoblastic differentiation of MC3T3-E1 cells, the cytokine profiles were analyzed in the cells treated with TNF-α in the presence and absence of quercetin (Fig. 1). TNF-α treatment significantly increased the production of IFN-γ compared to the control, such that 10 ng/ml TNF-α treatment stimulated the production of a 1.61-fold higher level of IFN-γ by MC3T3-E1 cells than by untreated control MC3T3-E1 cells (1.8 ng/ml) (Fig. 1A). By contrast, TNF-α-induced stimulation of INF-γ production was suppressed by treatment of quercetin. A prominent increase in IL-6 production by TNF-α and its suppression by quercetin treatment was also observed in MC3T3-E1 cells (Fig. 1B). For example, when MC3T3-E1 cells were treated with 10 ng/ml TNF-α, amount of IL-6 production was measured as 1.92 ng/ml, which represented a 11.3-fold increase compared to that of control cells (0.17 ng/ml). In contrast, when TNF-α-treated cells were incubated in the presence of 10 μM quercetin, IL-6 secretion by the cells was decreased to the level of 0.86 ng/ml. However, no significant changes were detected in the levels of IL-1β after treating MC3T3-E1 cells with TNF-α and/or quercetin (Fig. 1C). Both the TNF-α and quercetin did not induce any toxic effects in differentiated MC3T3-E1 cells during the treatment (data not shown). These results mean that quercetin plays a role as antiinflammatory chemicals and inhibits cytokine-mediated bone resorption.

TNF-α inhibited both differentiation and mineralization, as indicated by markers that are characteristic of the osteoblast phenotype, such as ALP activity (Fig. 2) and calcium deposition (Fig. 3), while this inhibition was slightly prevented by quercetin treatment. Catechin, one of the most common grape flavonols, caused a marked elevation of cell survival and ALP activity in MC3T3-E1 cells (Choi and Hwang, 2003). However, we failed to detect a dramatic preventive effect of quercetin on TNF-α-mediated inhibition of ALP activity and calcium deposition of MC3T3-E1 cells. By contrast, we found that quercetin treatment alone (10 μM) did not affect the ALP activity and calcium deposition in the cells. In order to elucidate the precise effects of quercetin on the differentiation and mineralization of osteoblasts, further detailed experiments about bone formation-related factors, such as type I collagen, osteocalcin, osteonectin, osteopontin, bone sialop-

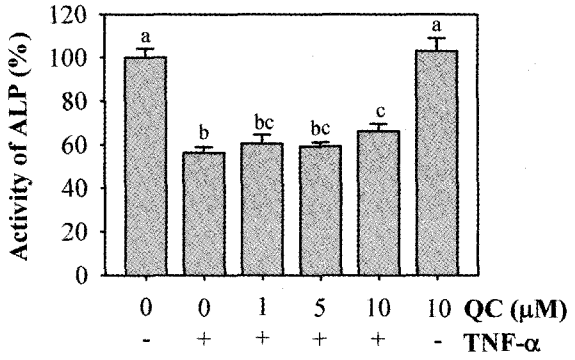

Fig. 2. Effect of quercetin on alkaline phosphatase (ALP) activity of MC3T3-E1 cells.

After the cells had reached confluence, quercetin was added to the cell cultures at various concentrations (1-10 μM) without or with 10 ng/ml TNF-α. At 7 days of the treatment, ALP activity was measured and different superscripts represent significant differences ($P < 0.05$) among groups by Duncan's multiple range test.

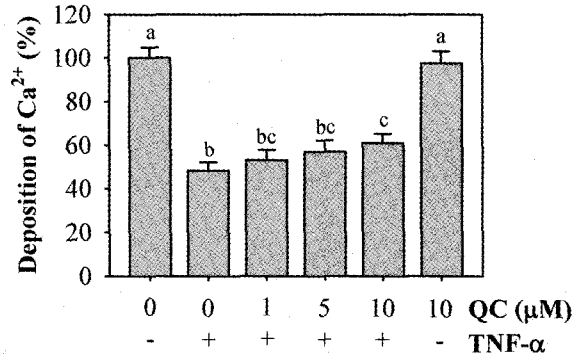

Fig. 3. Effects of quercetin on calcium deposition of MC3T3-E1 cells.

After the cells had reached confluence, quercetin was added to the cell cultures at various concentrations without or with 10 ng/ml TNF-α. At 7 days of the treatment, calcium deposition was measured and different superscripts represent significant differences ($P < 0.05$) among groups by Duncan's multiple range test.

rotein, and proteoglycans should be carried out (Marie, 2003).

Since it has been known that TNF-α produced nitric oxide in different cell types and the produced nitric oxide could induce apoptosis in osteoblasts (Chua *et al.*, 2002; Suh *et al.*, 2003), we determined whether quercetin inhibited the TNF-α-mediated nitric oxide production of MC3T3-E1 cells (Fig. 4). When a conditioned medium collected from the confluence MC3T3-E1 cells treated TNF-α for 2 days was measured, the concentration of nitrite (NO_2^-), a product of nitric oxide, was clearly increased, such that 10 ng/ml TNF-α treatment stimulated the production of nitrite to a 4.72-fold higher level by MC3T3-E1 cells than by untreated control cells (3.43 mmol/ml). However, quercetin treatment suppressed the

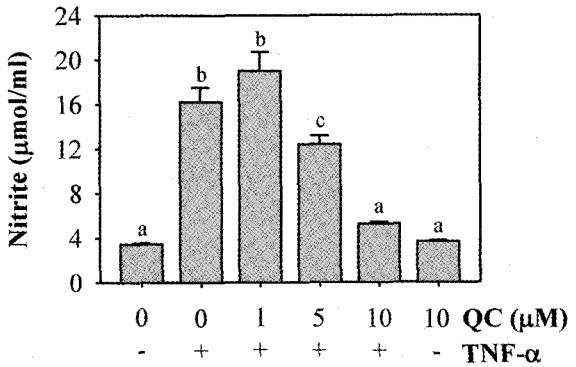

Fig. 4. Inhibitory effect of quercetin on TNF-α-induced production of nitric oxide in MC3T3-E1 cells.

The cells were treated with 10 ng/ml TNF-α in the presence of quercetin for 2 days. After incubation, culture supernatants were collected and nitrite contents were determined. The concentration of nitric oxide was assessed using a standard curve of $NaNO_2$. Different superscripts represent significant differences ($P < 0.05$) among groups by Duncan's multiple range test.

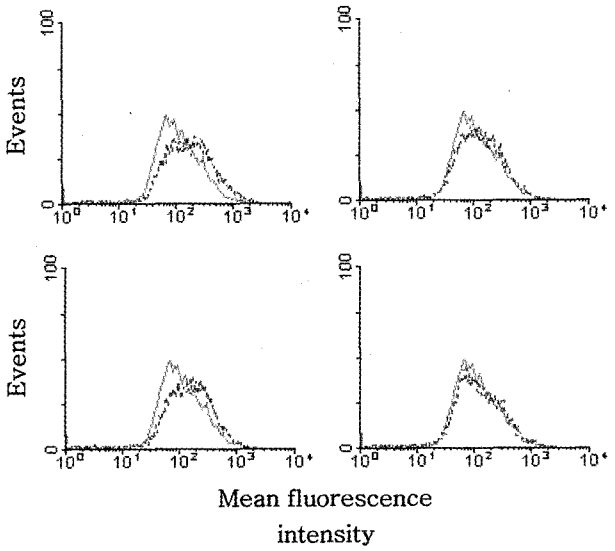

Fig. 5. Effects of quercetin on TNF-α-mediated production of reactive oxygen species (ROS) in MC3T3-E1 cells.

The cells were incubated for 24 h with 10 ng/ml TNF-α (A) in the presence of 5 (B) and 10 μM querectin (C). (D) is the cells treated with 10 μM quercetin alone. After incubation, DCFH-DA (25 μM) was added for an additional 20 min and the fluorescence distribution was analyzed. The same experiments were repeated three times and the figure is a representative result.

TNF-α-mediated production of nitric oxide in the cells. These results indicate that nitric oxide and/or its derivatives such as nitrite and peroxynitrite, which could

be generated by TNF-α, are not a direct inducer of growth inhibition and apoptotic cell death caused by TNF-α in proliferating osteoblastic cells (Notoya *et al.*, 2004). In addition, our findings suggest that inhibitory effects of quercetin on TNF-α-mediated production of inflammatory cytokines in MC3T3-E1 cells can be associated with its antioxidant property. The action of quercetin as antioxidant rather than prooxidant within the cells was further supported by the results that mean fluorescence intensity representing DCF content was clearly increased when the cells were treated with TNF-α (Fig. 5A), whereas the increase was reduced in the presence of quercetin in a dose-dependent manner (Figs. 5B and C).

In summary, our present study showed that quercetin inhibited inflammatory cytokine secretion induced by TNF-α in the chemical-induced differentiated MC3T3-E1 cells without any cytotoxic effects. In addition, quercetin significantly inhibited the TNF-α-mediated production of nitric oxide in the cells, although inhibitory effects of quercetin on TNF-α-mediated decreases of ALP activity and calcium deposition were not dramatic. In spite of increased knowledge regarding the benefits of quercetin, as potent antioxidant, its *in vivo* active form is still unclear. Moreover, it is unrevealed how quercetin acts on bone metabolism *in vivo*. Further detailed experiments should be performed to elucidate the precise contribution of quercetin to bone metabolism *in vivo*.

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