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Accelerating Effects of Quercetin on the TNF-α-Induced Apoptosis in MC3T3-E1 Osteoblastic Cells

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Abstract – Bioflavone quercetin is believed to play an important role preventing bone loss by affecting osteoclastogenesis and regulating many systemic and local factors including hormones and cytokines. This study examined how quercetin acts on tumor necrosis factor-alpha (TNF-α)-mediated apoptosis in MC3T3-E1 osteoblastic cells. Apoptosis assays revealed the dose-dependent acceleration of quercetin on TNF-α-induced apoptosis in MC3T3-E1 cells, which was demonstrated by the increased number of positively stained cells in the trypan blue staining and TUNEL assay, and the migration of many cells to the sub-G_0/G_1 phase in flow cytometric analysis. In particular, quercetin treatment alone increased the expression of p53 and p21 proteins in the cells. Consequently, this study showed that quercetin accelerates the TNF-α-induced apoptosis in MC3T3-E1 osteoblastic cells.
Keywords – Quercetin, TNF-α, MC3T3-E1 osteoblastic cells, apoptosis

Introduction

Bone cells and their interactions are quite sensitive to systemic and local factors such as hormones and cytokines. Among the many hormones and cytokines involved in regulating the bone metabolism, tumor necrosis factor-alpha (TNF-α) plays a key role in osteoporosis. TNF-α stimulates the osteoblasts to secrete other inflammatory cytokines such as interleukin (IL)-1 and IL-6, and prostaglandin E_2 (PGE_2) as well as TNF-α itself, which acts directly on osteoclasts to cause bone resorption (Franchimont *et al.*, 1997; Jilka, 1998; Glantschnig *et al.*, 2003). In addition, TNF-α induces the apoptosis of osteoblasts (Chua *et al.*, 2002; Suh *et al.*, 2003). Therefore, it is believed that an increase of TNF-α level and a decrease in the numbers of osteoblast cells via apoptosis might be responsible for the bone loss, and which can result in osteoporosis if these conditions persist.

Recently, there has been a global trend towards the use of natural bioactive compounds as chemoregulators of bone cells (Choi and Koo, 2003; Suh *et al.*, 2003; Gallagher *et al.*, 2004). Quercetin (3,3',4',5,7-pentahydroxyflavone) is a dietary flavone commonly found in plants, and has been reported to possess biological, pharmacological, and medicinal activity. For example, quercetin inhibits the osteoclastic resorption of bone *in vitro* (Wattel *et al.*, 2003). In addition, quercetin has a 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 reports 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, there is little information on the precise mechanism for quercetin on apoptosis of osteoblastic cells, particularly in the presence of inflammatory cytokines. Therefore, this study investigated how quercetin acts on the apoptosis induction of osteoblastic cells in the presence of TNF-α using MC3T3-E1, a mouse preosteoblast cell line.

Experimental

Chemicals and laboratory wares – Unless otherwise specified, chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. Quercetin (Q0125; Sigma Chemical

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Co.) was dissolved in ethanol immediately before use, and final concentration of ethanol did not exceed 0.1% (v/v) in any of the experiments.

Cell culture and treatment – Murine osteoblastic MC3T3-E1 cells (ATCC, CRL-2593) were cultured in a-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). The cells (1×10^5 cells/ml) were resuspended in 2 ml media and spread onto 35-mm culture dishes (Falcon, Becton Dickinson, NJ, USA). Cultures were switched with a fresh batch of the same medium twice a week. In order to determine the effects of quercetin on the apoptosis mediated by TNF-α (T7539; Sigma Chemical Co.), the medium was replaced with fresh medium containing various concentrations (0-10 μM) of quercetin and TNF-α (10 ng/ml), and incubated for various times (0-72 h).

Determination of cytotoxicity – A trypan blue exclusion assay was used to determine the level of cytotoxicity induced by the treatment with quercetin and/or TNF-α (Hongo *et al.*, 1986). Briefly, MC3T3-E1 cells were incubated in α-MEM supplemented with 10% FBS in the presence of 1 to 10 μM quercetin and/or 10 ng/ml TNF-α for various times. After incubation, the cells were stained with 0.4% trypan blue and about 100 cells were counted for each treatment. The level of cytotoxicity was calculated as follows: % cytotoxicity = [(total cells-viable cells)/total cells]×100.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay – After exposing MC3T3-E1 cells to 10 ng/ml TNF-α in the presence of quercetin (1-10 μM) for various times, the cells (2×10^6 cells) were fixed with 1% buffered formaldehyde (pH 7.5) on ice for 30 min. The cells were then washed with PBS, resuspended in 70% ice-cold ethanol, and kept at −20°C for 1 h. The cells were then rehydrated with PBS and incubated in TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. After 30 min incubation at 37°C, the reaction was blocked by transferring the cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin for 30 min. Finally, the cells were washed with PBS and observed using a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

Propidium iodide (PI) staining – The level of DNA fragmentation was also determined by flow cytometric analysis after propidium iodide (PI) staining. Initially, the suspension (2×10^6 cells) of quercetin- and/or TNF-α-treated cells was fixed with 80% ethanol at 4°C for 24 h and then incubated overnight at 4°C with 1 ml of PI staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 mg/ml PI in 1.12% sodium citrate). After staining, 1×10^4 cells were analyzed using the FACS Calibur® system (Becton Dickinson, San Jose, CA, USA).

Western blot analysis – Cell lysates were made in a lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 μg/ml of aprotinin, leupeptin, and pepstatin), and protein content was quantified using the Bradford (1976) method. Equal amounts of protein (50 μg/sample) were separated electrophoretically by 12% SDS-PAGE and blotted onto PVDF membranes (Bio-Rad, USA). The blots were probed with primary antibodies for either 2 h at room temperature or overnight at 4°C and incubated with horseradish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). Polyclonal antibody specific for p21 (SC-397) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies specific for p53 (OP03L-100UG) and a-tubulin were purchased from Oncogene and Sigma Chemical Co., respectively.

Statistical analyses – One-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results

Quercetin facilitates the TNF-α-induced apoptosis of MC3T3-E1 cells – Initially, we investigated the cytotoxic effect of quercetin on the cells by monitoring the level of trypan blue exclusion (Fig. 1A). As shown in the figure, TNF-α treatment increased the number of trypan blue stained cells. TNF-α-induced decrease in cell viability was further accelerated by the addition of quercetin. In contrast, quercetin treatment itself did not exert a substantial cytotoxic effect. There was also a marked increase in TUNEL positively stained cells with quercetin treatment. When MC3T3-E1 cells were treated with 10 ng/ml TNF-α alone for 72 h, the level of apoptosis was measured to be 52.4%, while 62.4% of the cells were apoptotic after adding 1 μM quercetin. More

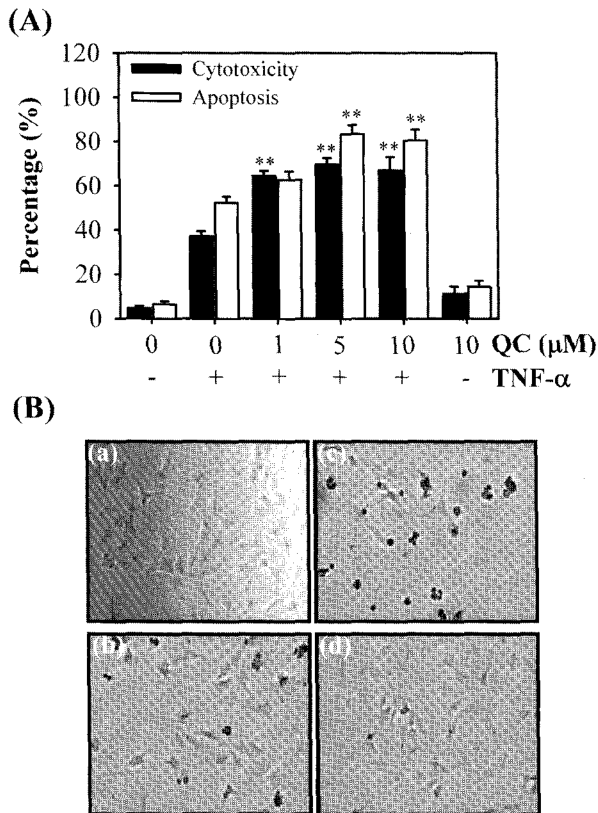

Fig. 1. Accelerating effect of quercetin on TNF-α-induced apoptosis in MC3T3-E1 cells (A) MC3T3-E1 cells were treated with different concentrations (0-10 μM) of quercetin in the presence or absence of 10 ng/ml TNF-α for 72 h, and the cells were then stained with trypan blue (open bar) or FITC-conjugated dUTP (closed bar) Each bar represents the mean ± SE of three separate experiments. $^{**}P < 0.01$ represents significant difference between the experiments and TNF-α treatment alone. (B) Cytotoxicity in the cells was also corroborated by observing cell morphology. The cells were treated without (a) or with 10 ng/ml TNF-α (b) in the presence of 10 μM (c) quercetin. (d) indicates the cells treated with 10 μM quercetin alone.

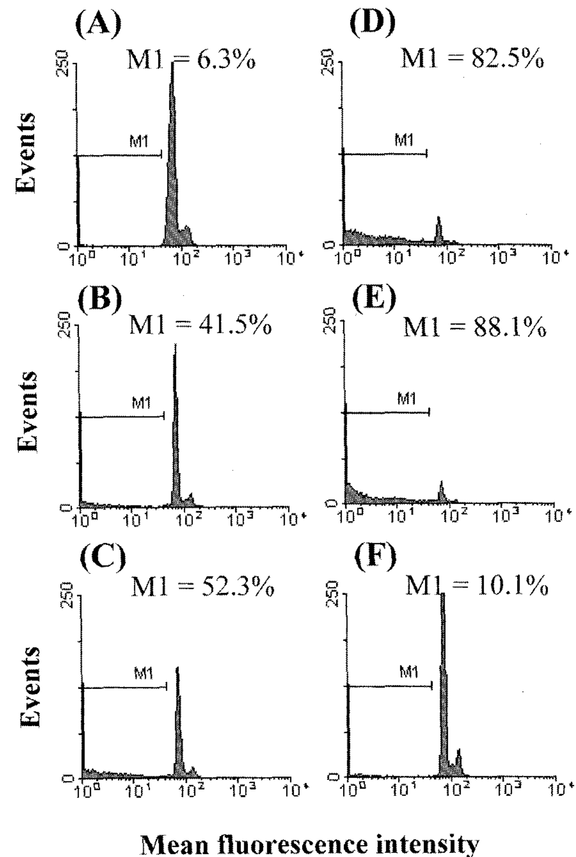

Fig. 2. Flow cytometric analyses of MC3T3-E1 cells after PI staining The cells were incubated for 72 h without (A) or with 10 ng/ml TNF-α in the absence (B) and presence of 1 (C), 5 (D), and 10 μM (E) quercetin. (F) is the cells treated with 10 mM quercetin only. The figures show a representative staining profile for 10,000 cells per experiment. M1 is the cell population defined as apoptotic.

than 80% of the cells were apoptotic after the cells were treated with 10 μM quercetin in the presence of 10 ng/ml TNF-α. The acceleration of TNF-α-induced cytotoxicity by the quercetin treatment was also corroborated by observing cell morphology (Fig. 1B). TNF-α-induced apoptosis and its facilitation by quercetin treatment was supported by PI staining (Fig. 2). In the absence of quercetin, apoptotic MC3T3-E1 cells were 41.5% of the total cell population after the TNF-α treatment (10 ng/ ml). However, the apoptotic cells were increased dose-dependently by the quercetin treatment, such that 52.3% and 88.1% of the cells were apoptotic when treated with 1 μM and 10 μM quercetin in the presence of 10 ng/ml TNF-α, respectively.

Cell cycle analysis revealed that after 72 h of exposure to 10 ng/ml TNF-α, about 53.6% of the cell populations were in the sub-G_0/G_1 phase, which indicates apoptotic cells, while about 31.2% and 7.2% of the cells were in the G_0/G_1 and G_2/M phases, respectively (Table 1). The addition of quercetin to the cells dose-dependently augmented the TNF-α-induced apoptosis. When 10 μM quercetin was added to the TNF-α-exposed cells, there were 89.2% of the total cell population in the sub-G_0/G_1 phase. However, when the cells were treated with quercetin alone (10 μM), there was an increase in the G_0/G_1 phase and a corresponding decrease in the S and G_2/M phases, compared with those of the untreated control cells.

Quercetin itself increases the expression of p53 and p21 in MC3T3-E1 cells – Because it is known that p53 modulates the cell cycle by activating p21, which induces a cell cycle arrest in G_0/G_1 phase, the effects of quercetin and/or TNF-α on the expression of p53 and p21 proteins were examined (Fig. 3A). Western blot analysis showed that the p53 expression level was not affected by TNF-α

Table 1. Effects of quercetin on the cell cycle distribution of MC3T3-E1 cells

Cell cycles	0	0	1	5	10	10	Quercetin (μM)
	–	+	+	+	+	–	TNF-α (ng/ml)
Sub-G_0/G_1	9.5±1.8	53.6±3.2	67.7±3.1	84.1±3.8	89.2±3.6	12.7±2.5	
G_0/G_1	63.1±3.8	31.2±2.7	21.8±2.4	10.5±3.4	7.7±2.8	76.1±3.1	
S	12.3±1.1	8.0±1.3	5.8±1.2	3.5±0.8	1.8±0.6	4.9±0.7	
G_2/M	15.1±1.0	7.2±1.1	4.7±0.6	1.9±0.3	1.3±0.3	6.3±1.2	

MC3T3-E1 cells were incubated with the indicated doses of quercetin for 72 h in the presence and absence of 10 ng/ml TNF-α. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of propidium iodide fluorescence data. Data represent the mean ± SD of experiments performed in triplicate.

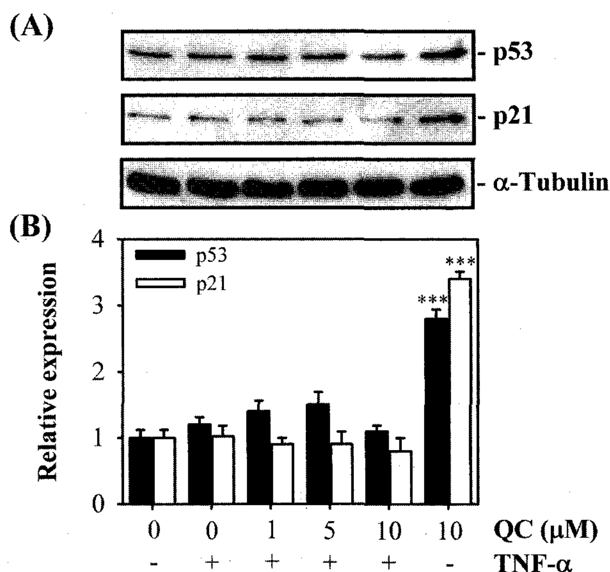

Fig. 3. Western blot analysis of MC3T3-E1 cells. MC3T3-E1 cells were incubated without or with 10 ng/ml TNF-α in the presence of 1 to 10 μM quercetin for 24 h. Cell lysates were prepared and analyzed by 12% SDS-PAGE followed by immunoblot analysis (A). A representative result from three independent experiments is shown. In addition, the expression level of p53 and p21 proteins was quantified and presented as the mean ± SE relative to control (relative expression = 1.0) (B). $^{***}P < 0.001$ represents significant difference between the experiments and untreated control values.

regardless of the addition of quercetin. In contrast, an approximate 2.8-fold increase in the p53 expression was observed in MC3T3-E1 cells exposed to 10 mM quercetin alone for 24 h (Fig. 3B). Similarly, the level of p21 expression in the cells was significantly elevated only when treated with quercetin itself. This can explain why quercetin itself arrests the cell cycle progression of MC3T3-E1 cells in the G_0/G_1 phase.

Discussion

The present study showed that the TNF-α-mediated cytotoxicity was not inhibited but facilitated by quercetin treatment in a dose-dependent manner, even though quercetin itself did not have any toxic effects. This suggests that in the presence of TNF-α, quercetin accelerates TNF-α-mediated cytotoxicity in growing MC3T3-E1 cells. Our previous findings demonstrated that quercetin inhibited TNF-α-induced cytokine secretion and nitric oxide production in differentiated MC3T3-E1 cells without any cytotoxic effects (Jeon *et al.*, 2005). In addition, quercetin reduced the TNF-α-induced inhibition of mineralization of the cells, indicating its ability preventing inflammatory cytokine-mediated bone loss. Considering these results, we believe that the acting mechanism of quercetin on bone cells may differ from the condition of the cells examined, *i.e.*, depending on exponentially growing or differentiated condition. Further detailed experiments will be needed to elucidate the precise mechanism of quercetin on bone formation and resorption.

TNF-α can induce apoptosis of osteoblastic MC3T3-E1 cells (Kitajima *et al.*, 1996; Suh *et al.*, 2003). Quercetin also induces apoptosis in a variety of tumor cells (Monasterio *et al.*, 2004; Son *et al.*, 2004; Ackland *et al.*, 2005). Apoptosis is the result of a highly complex cascade of cellular events that are characterized by chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing, and cell shrinkage (Allen *et al.*, 1997). As evidenced by the increase of TUNEL positive cells and the migration of many cells to sub-G_0/G_1 phase after PI staining, TNF-α treatment induced apoptosis of MC3T3-E1 cells, and this induction was further accelerated by the quercetin treatment.

Wild-type p53 modulates the cell cycle progression and cellular apoptosis (Adams and Kaelin, 1998). When DNA damage occurs, elevated p53 is translocated to the nucleus and activates the expression of p21, which induces cell cycle arrest in the G_0/G_1 phase. However, the cellular machinery leading to apoptosis can be induced if DNA damage is persisted and prolonged, or if p53 is overexpressed (Lowe *et al.*, 1993; Muller *et al.*, 1998).

Furthermore, p53 can induce apoptosis directly by activating *Bax* gene that contains p53-binding sites and encodes an apoptosis inducing factor (Miyashita and Reed, 1995). In this study, TNF-α treatment induced a clear increase in the number of cells in sub-G_0/G_1 phase, which represents apoptotic cells. TNF-α-induced apoptosis was further facilitated by the quercetin treatment in a dose-dependent manner. In contrast, quercetin itself (10 μM) induced cell cycle arrest of MC3T3-E1 cells in the G_0/G_1 phase. This was supported by Western blot analysis showing that quercetin, but not TNF-α, stimulated the expression of the p53 and p21 proteins in MC3T3-E1 cells. These results indicate that TNF-α-mediated apoptosis as well as its acceleration by quercetin is independent of p53.

Flavonoids have been reported to have inhibitory effects on PI3-kinase, protein kinase C, protein tyrosine kinase, mitogen-activated protein kinases, and some transcriptional factors (Bode and Dong, 2003). Moreover, this inhibition in turn has been shown to arrest cell growth and induce cell death in several cell lines (Yang *et al.*, 1998; Gamet-Payrastre *et al.*, 1999; Ishikawa and Kitamura, 2000; Lee *et al.*, 2003). As proven by the accumulation of MC3T3-E1 cells at the G_0/G_1 phase of the cell cycle, we assume that quercetin itself inhibits the signal transduction molecules involved in the cell cycle progression. Moreover, this inhibition might let the cells to further sensitive to the cytotoxic action of TNF-α. Therefore, we suggest that Fas activation, rather than p53 expression, is further associated with the TNF-α-induced apoptosis and its acceleration by quercetin in the cells. However, more detailed experiments will be needed to determine the precise mechanism of quercetin on the cell cycle arrest and apoptosis induction of osteoblasts in the absence and presence of inflammatory cytokines. In addition, it is unclear how quercetin acts, and it is not known if quercetin increases or decreases the bone mass *in vivo*. Additional experiments should be performed to elucidate the precise contribution of quercetin to the bone metabolism *in vivo* and *in vitro*.

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