

Quercetin-mediated apoptosis via activation of the mitochondrial-dependent pathway in MG-63 osteosarcoma cells

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Received February 7, 2011; Accepted July 6, 2011

DOI: 10.3892/mmr.2011.533

Abstract. Quercetin (a natural polyphenolic compound) is a polyphenolic flavonoid compound found in a variety of plants. It has been demonstrated to exert cytostatic activity against a variety of human cancer cell lines, including the human osteosarcoma cell line, MG-63. However, its effects on osteosarcoma cell apoptosis are still undefined. The present study was undertaken to examine the effect of quercetin on cell viability, apoptosis and mitochondrial membrane potential, and to determine the molecular mechanism of quercetin-induced apoptosis by investigating the expression of Bcl-2 family proteins (Bcl-2, Bax), cytochrome C, caspase-9 and caspase-3 in MG-63 cells. We found that quercetin suppressed the viability of MG-63 cells in a dose- and time- dependent manner. Furthermore, we observed that quercetin induced the loss of mitochondrial membrane potential, upregulated the expression of the proapoptotic proteins, Bax and cytochrome C, and activated caspase-9 and caspase-3, and downregulated the expression of antiapoptotic protein, Bcl-2. These data suggest that quercetin may induce apoptosis via the mitochondrial-dependent pathway in MG-63 cells.

Introduction

Osteosarcoma (OS) is the third most common cancer in childhood and adolescents, and the most common primary malignant bone tumor (1). With the development of new chemotherapy protocols, surgical techniques and radiological staging, the 5-year survival and cure rates have increased to 60-80% in patients with localized disease (2). However, major problems associated with chemotherapy still remain, particularly the frequent development of drug resistance (3). Moreover, chemo-

therapy may induce DNA mutations in normal cells. Hence the challenge of seeking new therapeutic approaches that can further improve the efficiency and alleviate the adverse effects is clear and urgent (4). Epidemiological studies have shown that regular consumption of tea, vegetables and fruits is associated with reduced risk of cancer (5). Quercetin with a molecular formula of $C_{15}H_{10}O_7$ (Fig. 1), is an abundant flavonoid in tea, fruits and vegetables (6). Quercetin has been shown to induce cell death by apoptosis in many human cancer cell lines, such as hepatoma, oral, leukemia, lung and colon cancer cell lines (7,8).

Apoptotic cell death is an innate cellular response to eliminate abnormal or redundant cells in mammals and hence is crucial for mammal development and tissue homeostasis (9). The disturbed regulation of this vital process represents a major causative factor in the pathogenesis of cancers, including OS (10,11). The mitochondrial-dependent pathway is one of the apoptotic pathways in mammalian cells. Mitochondrial membrane permeabilization, accompanied by the collapse of electrochemical gradient across the mitochondrial membrane causes the release of catabolic hydrolases and activators of such enzymes (including those of caspases) from the mitochondria, leading to cell apoptosis (12,13). Bcl-2 family proteins serve as critical regulators of this pathway through their influence on mitochondrial outer membrane permeabilization (MOMP) following homo- or hetero-association (14). Among Bcl-2 family proteins, pro-apoptotic members, such as Bax and Bak increase MOMP during apoptosis, releasing apoptogenic proteins, such as cytochrome C and Diablo/Smac that trigger apoptosis by activating caspases and nucleases; whereas anti-apoptotic members, such as Bcl-2 may bind to activate Bax to decrease membrane permeability (15). The regulation of active anti- and pro-apoptotic Bcl-2 family members is a key for determining the fate of cells, and disturbance of the normal apoptotic program due to alteration of the ratio by aberrant expression of these proteins may lead to various apoptosis-related diseases (16,17). In addition, Bcl-2 overexpression, widely found in a variety of cancers, suppresses apoptosis and confers resistance to anticancer drugs. Therefore, the induction of apoptosis through the mitochondrial-dependent pathway has been one of the goals of anti-cancer chemotherapy.

In this study, we evaluated the effect of quercetin on the apoptosis of the human osteosarcoma cell line, MG-63, and investigated the possible underlying molecular mechanisms.

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Key words: osteosarcoma, apoptosis, quercetin, mitochondria

We found that the quercetin-induced apoptosis of MG-63 cells was accompanied by the loss of mitochondrial membrane potential ($\Delta\psi_m$), downregulation of Bcl-2, upregulation of Bax and cytochrome C, and caspase-9 and caspase-3 activation. Our data suggest that quercetin can induce MG-63 cell apoptosis via activation of the mitochondrial-dependent pathway.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) and TRIzol reagent, caspase-9 and caspase-3 colorimetric protease assay kits were provided by Invitrogen (Grand Island, NY, USA). SuperScript II reverse transcriptase was obtained from Promega (Madison, WI, USA). Antibodies against Bcl-2, Bax, cytochrome C and β -actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). The fluorescein isothiocyanate (FITC)-conjugated Annexin V apoptosis detection kit was provided by Becton-Dickinson (San Jose, CA, USA). Quercetin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of quercetin were prepared by dissolving the quercetin powder in DMSO to a concentration of 1 M, and stored at -20°C . The working concentrations of quercetin were made by diluting the stock solution with the culture medium. The final concentration of DMSO in the medium was $<0.5\%$.

Cell culture. Human osteosarcoma cell line MG-63 cells from the American Type Culture Collection (Manassas, VA) were maintained at 37°C in an atmosphere with $5\% \text{CO}_2$ with DMEM, supplemented with $10\% \text{ (v/v)}$ FBS, 100 U/ml penicillin, and $100 \mu\text{g/ml}$ streptomycin. MG-63 cells were subcultured at $80\text{--}90\%$ confluency. The cells used in this study were subjected to no more than 20 cell passages.

Cell viability by MTT assay. The cells were cultured in 96-well plates at a concentration of 1×10^4 cells/well. Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cells were treated with quercetin at various final concentrations ($20, 40, 80, 160, 240$ and $320 \mu\text{M}$, respectively), and the vehicle control cells treated with $0.5\% \text{ DMSO}$ for 48 h. In some experiments, cells were treated with $160 \mu\text{M}$ of quercetin for different periods of time. After treatment, $10 \mu\text{l}$ MTT [5 mg/ml in phosphate buffered saline (PBS)] were added to each well, and the samples were incubated at 37°C for 4 h. The purple-blue MTT formazan precipitate was dissolved in $100 \mu\text{l}$ DMSO and cells were shaken for 10 min. The absorbance was measured at 490 nm using an ELISA reader (BioTek, Model EXL800, USA).

Observation of morphological changes. MG-63 cells were cultured in 6-well plates at a concentration of 2×10^5 cells/well. The cells were treated with various concentrations of quercetin for 48 h. MG-63 cell morphology was observed using a phase-contrast microscope (Olympus, Japan). The photographs of MG-63 cells were taken at a magnification of $\times 100$.

Measurement of apoptosis by flow cytometry analysis with Annexin V/propidium iodide (PI) staining. After treatment with

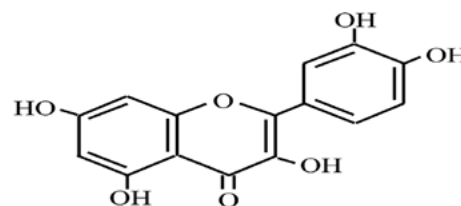


Figure 1. The chemical structure of quercetin.

various concentrations of quercetin, the apoptosis of MG-63 cells was determined by flow cytometry analysis using a fluorescence-activated cell sorting (FACS)calibur (Becton-Dickinson, CA, USA) with Annexin V-fluorescein isothiocyanate (FITC)/PI staining. The staining was performed according to the manufacturer's instructions. Early apoptotic cells were determined by Annexin V-positivity and PI-negativity, while late apoptotic cells were determined by Annexin V-positivity and PI-positivity.

Detection of $\Delta\psi_m$ by flow cytometry analysis with JC-1 staining. JC-1 is a cationic dye that can be used as an indicator of mitochondrial potential. It exhibits mitochondrial potential-dependent accumulation, which can be detected by a fluorescence emission shift from green to red. After treatment with various concentrations of quercetin, MG-63 cells were trypsinized, resuspended in 1 ml of medium and incubated with $10 \mu\text{g/ml}$ of JC-1 (Invitrogen) at 37°C for 30 min. After JC-1 staining, red and green fluorescence emissions were analyzed by flow cytometry.

RNA extraction and RT-PCR analysis. MG-63 cells were cultured in 6-well plates at a concentration of 2×10^5 cells/well and treated with various concentrations of quercetin for 48 h. Total RNA from MG-63 cells was isolated with TRIzol reagent (Invitrogen). Oligo(dT)-primed RNA ($5 \mu\text{g}$) was reverse transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Bcl-2 and Bax by PCR with Taq DNA polymerase (Fermentas). β -actin was used as the internal control. The primers used for the amplification of Bcl-2, Bax and β -actin transcripts were as follows: Bcl-2 (310 bp) forward, $5'\text{-CGA CTT CGC CGA GAT GTC CAG CCA G-3'}$ and reverse, $5'\text{-ACT TGT GGC CCA GAT AGG CAC CCA G-3'}$; Bax (289 bp) forward, $5'\text{-CTG ACA TGT TTT CTG ACG GC-3'}$ and reverse, $5'\text{-TCA GCC CAT CTT CTT CCA GA-3'}$; β -actin (285 bp) forward, $5'\text{-AGC GAG CAT CCC CCA AAG TT-3'}$ and reverse, $5'\text{-GGG CAC GAA GGC TCA TCA TT-3'}$.

Western blot analysis. MG-63 cells were cultured in 6-well plates at a concentration of 2×10^5 cells/well. After treatment with quercetin at various concentrations for 48 h, cells were lysed with mammalian cell lysis buffer (M-PER; Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (EMD Biosciences and Sigma Chemical, USA, respectively), and protein concentrations were determined by the BCA assay using bovine serum albumin as the standard. The samples ($20 \mu\text{g}$ for each) were loaded and separated by electrophoresis on $12\% \text{ SDS-polyacrylamide}$ gels

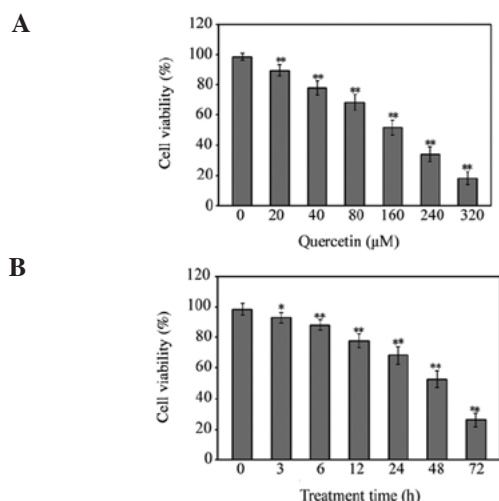


Figure 2. Effect of quercetin on cell viability of MG-63 cells. (A) MG-63 cells were treated with the indicated concentrations of quercetin for 48 h. (B) Cells were treated with 160 μ M of quercetin for the indicated time-periods. Cell viability was determined by the MTT assay. The data were normalized to the viability of the control cells (100%). Data are averages \pm SD (error bars), * P <0.05, ** P <0.01, compared to the control cells.

under a reducing condition using 200 V for 1 h. After electrophoresis, the proteins were transferred to PVDF membranes in a Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 0.05% w/v SDS, 10% v/v methanol) using a semi-dry blotting system, and incubated with antibodies against Bcl-2, Bax, cytochrome C and β -actin (1:1000) overnight at 4°C with rocking. After PVDF membranes were washed in TBST, secondary HRP-conjugated antibodies were added at 1:2000 dilution for 1 h at room temperature and the PVDF membranes were washed again in TBST. Blots were developed using Super Signal Pico Substrate, and images were taken using a Kodak image station. Protein bands were analyzed using the Fluor-s Gel Imaging Analysis System and normalized to β -actin in the sample.

Analysis of caspase-9 and caspase-3 activation. MG-63 cells were cultured in 6-well plates at a concentration of 2×10^5 cells/well and treated with various concentrations of quercetin for 48 h. The activities of caspase-9 and caspase-3 were determined by a colorimetric assay using the caspase-9 and caspase-3 activation kits (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were lysed with provided lysis buffer for 30 min on ice and centrifuged at $16000 \times g$ for 10 min. The protein concentration was measured by the BCA assay with bovine serum albumin as the standard. Then, 100 μ g of the protein were incubated with 50 μ l of the colorimetric tetrapeptides, Leu-Glu-His-Asp (LEHD)-p-nitroaniline (pNA) (specific substrate of caspase-9) or Asp-Glu-Val-Asp (DEAD)-pNA (specific substrate of caspase-3) at 37°C for 2 h. The samples were read at 405 nm in an ELISA reader (BioTek, Model EXL800, USA).

Statistical analysis. Data were analyzed using the SPSS package for Windows (Version 13.0). The quantitative data were expressed as the means \pm standard deviation (SD). Statistical analysis of the data was performed using the Student's t-test

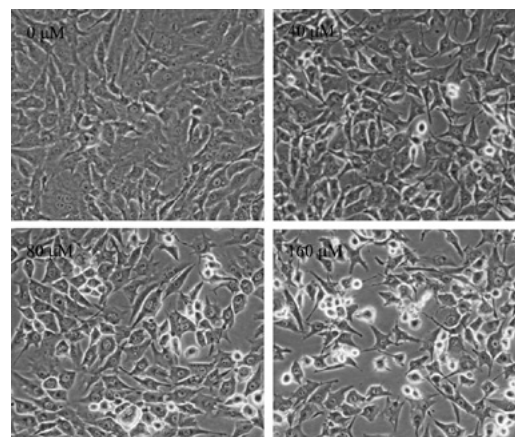


Figure 3. Effect of quercetin on the morphological changes of MG-63 cells (x100). MG-63 cells were treated with the indicated concentrations of quercetin for 48 h and morphological changes were observed using phase-contrast microscopy.

and ANOVA. Differences with P -values of <0.05 were considered statistically significant.

Results

Quercetin reduces the viability of MG-63 cells. The effect of quercetin on the viability of MG-63 cells was determined by MTT assay. As shown in Fig. 2A, after 48 h, the cell viability was reduced to $89.47 \pm 3.63\%$, $77.65 \pm 4.77\%$, $68.14 \pm 5.29\%$, $51.48 \pm 4.96\%$, $33.93 \pm 4.83\%$ and $18.27 \pm 4.42\%$ when cells were treated with quercetin at the final concentrations of 20, 40, 80, 160, 240 and 320 μ M, respectively, significantly lower than that of the control cells ($98.28 \pm 2.44\%$) (P <0.01), indicating that the inhibitory effects were dose-dependent and the estimated half-maximal inhibitory concentration (IC₅₀) value was 160 μ M. Hence, 160 μ M was selected in the following experiments. As shown in Fig. 2B, quercetin reduced cell viability in a time-dependent manner.

Quercetin induces morphological changes in MG-63 cells. The effect of quercetin on MG-63 cell morphology was evaluated by phase-contrast microscopy. As shown in Fig. 3, control MG-63 cells appeared as densely disorganized multilayers, whereas many of the cells became shrunken and round-shaped, and detached from each other or floated in the medium after incubation with various concentrations of quercetin for 48 h, suggesting that quercetin can reduce the viability of MG-63 cells.

Quercetin induces apoptosis of MG-63 cells. To further investigate whether the suppressive effect of quercetin on cell viability is due to apoptosis, MG-63 cells were subjected to FACS analysis after Annexin-V/PI staining. In the FACS diagram, LL (Annexin V/PI double-negative population) indicates viable cells; LR or UR (Annexin V-positive/PI-negative or Annexin V/PI double-positive population) represents cells undergoing early or late apoptosis respectively. As shown in Fig. 4A and C, the percentages of cells undergoing apoptosis (including the early and late apoptotic cells) were $21.28 \pm 3.07\%$, $33.23 \pm 5.78\%$ and $48.52 \pm 5.33\%$ when the final concentrations

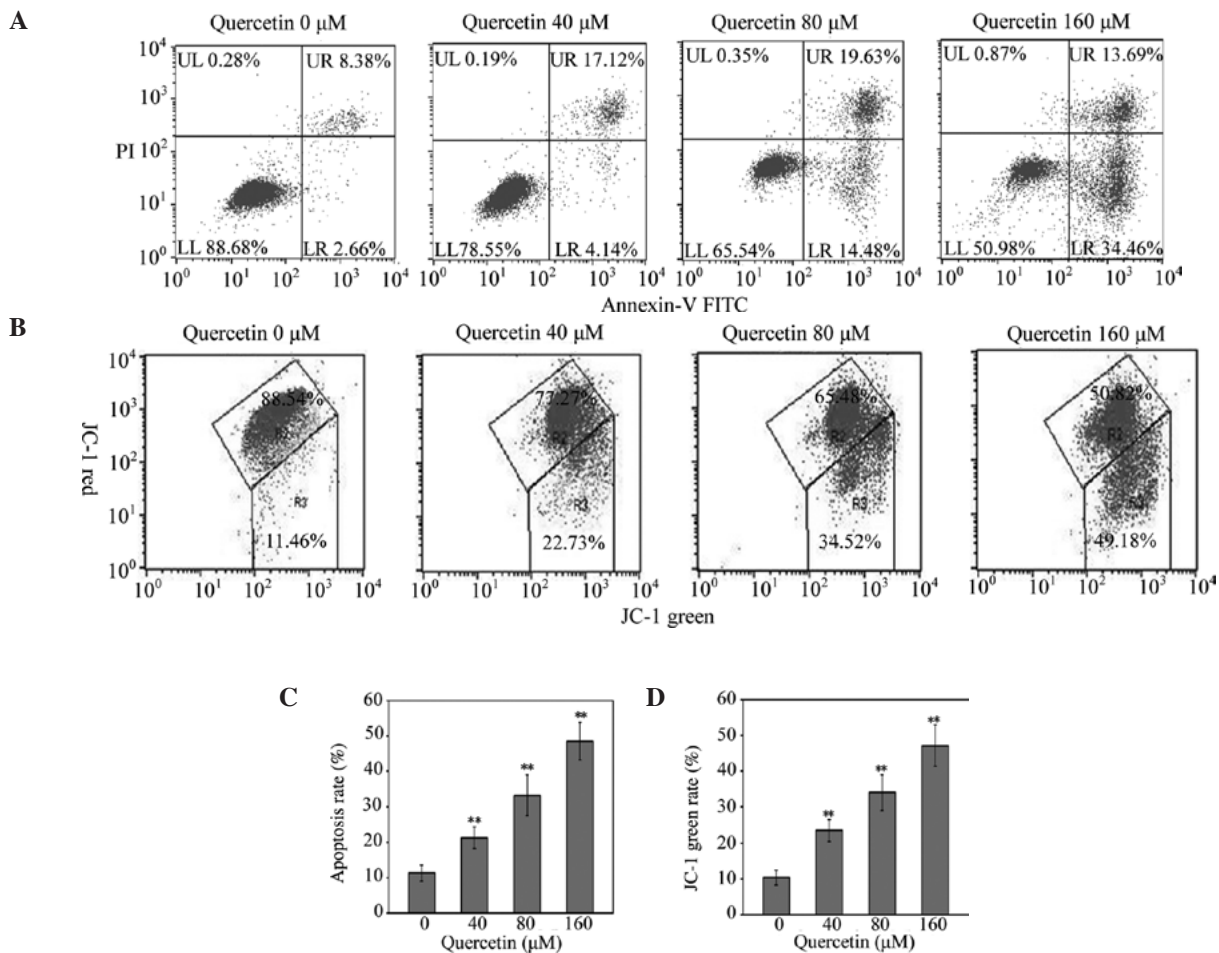


Figure 4. Effect of quercetin on the apoptosis of MG-63 cells. (A) After treatment with the indicated concentrations of quercetin for 48 h, MG-63 cells were collected and stained with Annexin V/PI followed by FACS analysis. Representative FACS analysis scatter-grams of Annexin V/PI staining displays four different cell populations labeled as: LL (lower left), double-negative stained cells indicating live cell population; LR (lower right), Annexin V-positive/PI-negative stained cells representing early apoptosis; UR (upper right), Annexin V/PI double-positive stained cells displaying late apoptosis; UL (upper left), Annexin V-negative and PI-positive stained cells showing dead cells. (B) MG-63 cells were treated with the indicated concentrations of quercetin for 48 h and stained with JC-1. The mean JC-1 fluorescence intensity was detected using FACS analysis. (C) Quantification of apoptosis in MG-63 cells by FACS analysis. The data shown are the averages \pm SD (error bars), ** $P < 0.01$, significant vs. control cells. (D) Quantification of the loss of $\Delta\psi_m$ in MG-63 cells by FACS analysis. The data shown are the averages \pm SD (error bars), ** $P < 0.01$, compared to the control cells.

of quercetin were 40, 80 and 160 μM , respectively, significantly higher than the control cells ($11.26 \pm 2.23\%$) ($P < 0.01$). This indicates that quercetin induces MG-63 cell apoptosis in a dose-dependent manner.

Quercetin induces the loss of $\Delta\psi_m$. To confirm the pro-apoptotic effect of quercetin, we then investigated the influence of quercetin on the loss of $\Delta\psi_m$, a typical feature of apoptosis. Change in $\Delta\psi_m$ was detected by FACS analysis after JC-1 staining. JC-1 selectively enters the mitochondria and forms J-aggregates with intense red fluorescence (590 nm) in healthy cells. In apoptotic cells, the altered mitochondrial transmembrane potential causes JC-1 to remain in the cytoplasm in monomeric form, showing green fluorescence (525 nm). These fluorescence changes can be detected by FACS using JC-1 green and red channels. As shown in Fig. 4B and D, JC-1 fluorescence shifted from a JC-1-green-bright/JC-1-red-bright signal in the control MG-63 cells to a JC-1-green-bright/JC-1-red-dim signal in the cells treated with quercetin in a dose-dependent fashion. The percentages of JC-1-green-bright cells with 40 μM ($23.53 \pm 3.10\%$), 80 μM ($34.05 \pm 4.96\%$) and

160 μM ($47.18 \pm 5.67\%$) of quercetin were significantly higher than the control cells ($10.38 \pm 2.12\%$, $P < 0.01$), indicating the quercetin-induced loss of $\Delta\psi_m$ in MG-63 cells.

Quercetin decreases the expression of anti-apoptotic Bcl-2 and increases pro-apoptotic Bax and cytochrome C. Bcl-2 family proteins, including anti-apoptotic members, such as Bcl-2 and pro-apoptotic members, such as Bax and cytochrome C, play important roles in mitochondrial-mediated apoptosis regulation. To further investigate how quercetin induces cancer cell apoptosis, the mRNA transcription and protein expression of Bcl-2, Bax and cytochrome C in quercetin-treated cells were evaluated by RT-PCR and Western blot analysis, respectively. The results of the RT-PCR assay showed that quercetin treatment profoundly reduced the transcription of the Bcl-2 gene and increased Bax transcription in MG-63 cells compared to the control cells ($P < 0.01$) (Fig. 5A-C). The results were confirmed by Western blot analysis. In addition, cytochrome C protein expression was significantly increased by quercetin treatment ($P < 0.01$, vs. control) (Fig. 6A-D). This suggests that quercetin induces mitochondrial-dependent apoptosis in

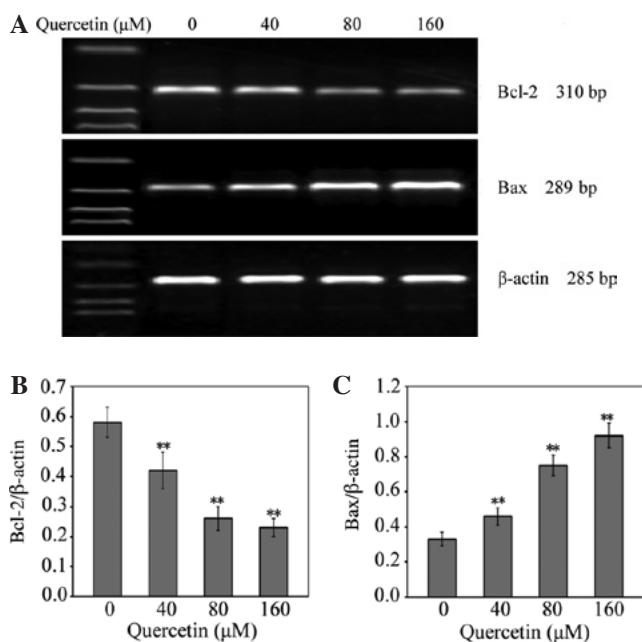


Figure 5. Effect of quercetin on the mRNA expression of Bcl-2 and Bax in MG-63 cells. MG-63 cells were treated with the indicated concentrations of quercetin for 48 h. (A) The mRNA expression of Bcl-2 and Bax in quercetin-treated and control cells was determined by RT-PCR. β-actin was used as the internal control for the RT-PCR assays. Quantification of RT-PCR analysis, the data shown are the averages \pm SD (error bars), **P<0.01, compared to the control cells. (B) The mRNA expression of Bcl-2 in quercetin-treated and control cells. (C) The mRNA expression of Bax in quercetin-treated and control cells.

MG-63 cells via regulating the expression of the Bcl-2 family proteins.

Quercetin treatment results in increased activities of caspase-9 and caspase-3. The mitochondrial membrane permeabilization results in the release of numerous apoptogenic proteins from the mitochondria, triggering the activation of caspase-9 and caspase-3, and eventually leading to apoptosis. To investigate the downstream effectors in the apoptotic signaling pathway, the activation of caspase-9 and caspase-3 was detected by a colorimetric assay using the specific chromophores, LEHD-pNA (specific substrate of caspase-9) and DEVD-pNA (specific substrate of caspase-3), respectively. As shown in Fig. 7A and B, quercetin treatment significantly and dose-dependently increased the activities of caspase-9 and caspase-3 in MG-63 cells compared to the control cells (P<0.05 and P<0.01, respectively). Taken together, these results suggest that quercetin promotes MG-63 cell apoptosis via the mitochondrial-dependent pathway.

Discussion

Our study demonstrates that quercetin induces the loss of Δψ_m, upregulates the expression of the proapoptotic proteins, Bax and cytochrome C, and downregulates the expression of the antiapoptotic protein, Bcl-2, resulting in the increased activity of caspase-9 and caspase-3. Hence, quercetin reduces the viability of MG-63 cell at least via the mitochondrial-dependent pathway.

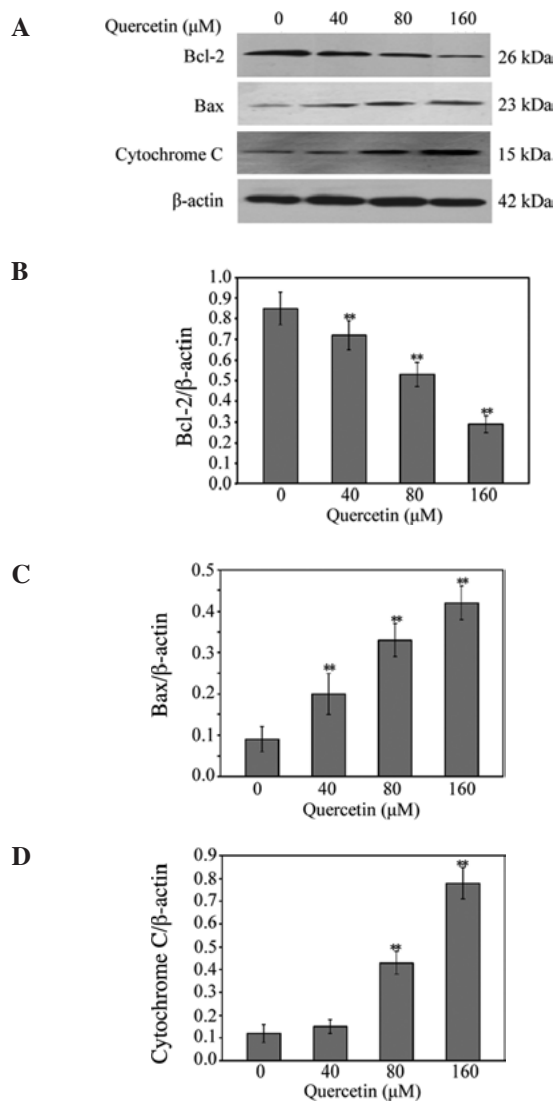


Figure 6. Effect of quercetin on the protein expression levels of Bcl-2, Bax and cytochrome C in MG-63 cells. (A) The protein expression levels of Bcl-2, Bax and cytochrome C were analyzed by Western blot analysis. β-actin was used as the internal control for the Western blot analysis. Quantification of Western blot analysis. The data shown are the averages \pm SD (error bars), **P<0.01, significant vs. control cells. (B) The protein expression levels of Bcl-2 in quercetin-treated and control cells. (C) The protein expression levels of Bax in quercetin-treated and control cells. (D) The protein expression levels of cytochrome C in quercetin-treated and control cells.

Tumor cells are characterized by an unregulated increase in cell proliferation and/or a reduction in cell apoptosis. Moreover, disrupted apoptosis contributes to drug resistance of tumor cells, which has become a significant obstacle for the successful management of patients with malignant tumors, including OS (18). The growing amount of evidence from studies on tumor cell cultures and animal tumor models demonstrates that a large number of natural products from the diet can sensitize tumor cells to anti-cancer therapies (19,20). For cancer prevention and chemotherapy, plant-derived natural products are an invaluable treasure and worthy to be further explored.

Quercetin, one of the major dietary flavonoids found in various teas, vegetables and fruits, has been reported to exert a broad range of anticancer effects. It can selectively inhibit the proliferation of tumor cells and induce cell apoptosis (21,22).

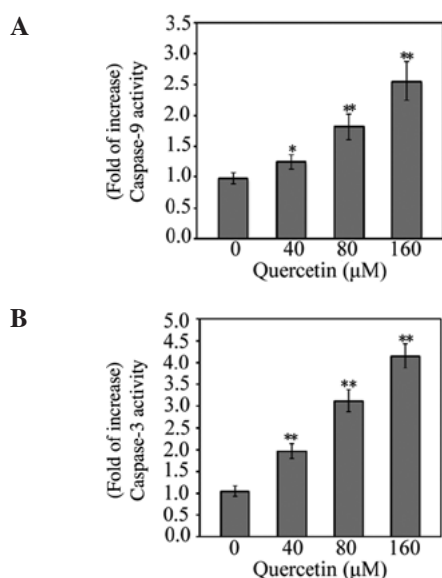


Figure 7. Effect of quercetin on the activity of caspases in MG-63 cells. MG-63 cells were treated with the indicated concentrations of quercetin for 48 h. Caspase-9 and caspase-3 activities were determined by a colorimetric assay. The data were normalized to the caspase activities of the control cells and represented as the fold of the control. The data shown are the averages \pm SD (error bars). * $P<0.05$, ** $P<0.01$, compared to the control cells. (A) The activity of caspase-9 in quercetin-treated and control MG-63 cells. (B) The activity of caspase-3 in quercetin-treated and control MG-63 cells.

Suh *et al* reported that the antiproliferative action of quercetin human osteosarcoma cells appeared to be linked to apoptotic cell death based on increase in the sub-G(1) apoptotic cell population (9). However, the underlying molecular mechanism is not fully understood.

Apoptosis is triggered by two different signals. The extrinsic apoptotic pathway involves death receptors, which respond mainly to extracellular stimuli. The intrinsic apoptotic pathway involves the mitochondria that are activated by modulators within the cell itself (23,24). Both pathways eventually lead to the activation of caspases and nucleases, resulting in cell death. The mitochondria continuously collect information on various aspects of signal transduction cascades and cellular metabolism, process this information, then decide on the fate of cells and participate in the regulation of cell death. MOMP is a key commitment step in the induction of cellular apoptosis, as it is often required for the activation of the caspase proteases that cause apoptotic cell death (25). During the process of MOMP, the electrochemical gradient across the mitochondrial membrane collapses, hence the loss of $\Delta\psi_m$ is a hallmark for apoptosis. Our data clearly show that treatment with quercetin leads to the collapse of $\Delta\psi_m$ in MG-63 cells.

Mitochondrial-dependent apoptosis upstream of caspase activation is regulated by members of the Bcl-2 family (26). Apoptosis-associated MOMP is known to require pro-apoptotic Bax-like proteins, in the regulation of the formation of pores in the mitochondria. Anti-apoptotic Bcl-2-like proteins in mitochondrial morphogenesis are functionally distinct from their role in apoptosis. Therefore, the ratio of Bax to Bcl-2 is critical for determining the release of many apoptogenic proteins from the mitochondrial intermembrane space, such as cytochrome C (27,28). Cytochrome C can activate caspase-9.

The activated caspase-9 cleaves and activates executioner caspases, such as caspase-3, then drives the caspase cascade and the cell death mechanism (29).

In this study, we demonstrate that quercetin dose-dependently upregulates Bax expression and downregulates Bcl-2 expression in MG-63 cells. This indicates that quercetin induces apoptosis by affecting the ratio of Bax/Bcl-2. This results in enhanced cytochrome C expression and increases the activities of caspase-9 and caspase-3.

In conclusion, our data demonstrate that quercetin induces MG-63 cell apoptosis via the mitochondrial-dependent pathway. These results indicate that quercetin could be a potential novel therapeutic agent for the treatment of OS. Further studies are required in order to find out whether quercetin can synergize with other chemotherapy drugs.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant no. 30772697).

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