Quercetin Potentiates Apoptosis by Inhibiting Nuclear Factor-kappaB Signaling in H460 Lung Cancer Cells

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The herbal flavonoid quercetin inhibits the growth of various cancer cells, but how it affects human cancer cells, particularly lung cancer cells, is unclear. We investigated the anticancer activity of quercetin and the underlying molecular mechanisms in non-small cell lung cancer (NSCLC) cells. Quercetin strongly inhibited cell proliferation, and increased sub-G1 and apoptotic cell populations regardless of p53 status. Quercetin-induced apoptosis was verified by caspase cleavage, Hoechst staining, trypan blue exclusion, and DNA fragmentation assays. Microarray analysis using H460 cells indicated that quercetin increased the expression of genes associated with death receptor signaling, tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAILR), caspase-10, interleukin (IL) 1R DNA fragmentation factor 45 (DFF45), tumor necrosis factor receptor (TNFR) 1, FAS, inhibitor of kappaBalptha (IxBa) and cell cycle inhibition growth arrest and DNA-damage inducible 45 (GADD45), p21(133), but decreased the expression of genes involved in nuclear factor (NF)-kappaB activation (NF-xB, IKKa). Further validation assays confirmed that quercetin inhibited growth by suppressing NF-xB and by increasing the expression of death receptors and cell cycle inhibitors. Taken together, these findings suggest that quercetin may be useful in the prevention and therapy of NSCLC.

Key words quercetin; lung cancer; apoptosis; death receptor; nuclear factor-kappaB pathway

Lung cancer is the leading cause of cancer death in many developed countries because of its poor prognosis. Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases, of which 70% show advanced-stage disease at the time of diagnosis.1, 2 Although various chemical drugs have been developed,3–4 frequent chemoresistance and side effects in patients with NSCLC require the development of new preventive and therapeutic agents. High intake of flavonoids from fruits and vegetables is associated with a low risk of various cancers,5–10 including lung cancer.11, 12 Of these polyphenolic flavonoids, quercetin is readily found in fruits, vegetables, green and black tea, and various medical plants such as Euonymus alatus (Thunb.) Sieb. Quercetin can act as either an antioxidant or a prooxidant depending on its concentration (1–40 µM or 40–100 µM, respectively).13, 14 Numerous studies have reported a broad range of pharmacological properties of quercetin, including benefits for inflammation,15 atherosclerosis,16 hypertension,17 and neurodegeneration.18, 19 In addition to these beneficial effects, quercetin plays preventative and therapeutic roles in various types of cancer and cancer cells.20–25 However, the anticancer effect of quercetin in NSCLC cells has been rarely addressed, and the underlying molecular mechanism remains to be determined.

Here, we investigated the growth inhibitory role of quercetin in three NSCLC cell lines and found that its effect is p53-independent and triggered by apoptosis. Subsequent microarray analysis using H460 cells indicated that quercetin enhances the expression of genes associated with death receptor signaling and cell cycle inhibition, but decreases the expression of genes involved in nuclear factor (NF)-xB activation. In contrast to down-regulated NF-xB expression, its inhibitor of kappaBalptha (IxBa) was upregulated by quercetin at both the mRNA and protein levels. The inhibitory role of quercetin in the NF-xB pathway was further demonstrated by suppressed NF-xB nuclear translocation and reduced NF-xB transcriptional activity following quercetin treatment.

MATERIALS AND METHODS

Reagents and Chemicals Quercetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), Hoechst 33342, 7-amino-actinomycin D (7-AAD), trypan blue, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell Culture The human NSCLC cell lines H460 (p53 wild-type (wt)), A549 (p53 wt), and H1299 (p53 null) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and an antibiotic-antimycotic mixture (all from Invitrogen, Carlsbad, CA, U.S.A.) in a 5% CO2 atmosphere at 37°C. The cells were treated with quercetin for the indicated periods of time.

Cell Viability Assay To determine the effect of quercetin on cell viability, IC50 values were measured by MTT assay, as described previously.26 H460, H1299, and A549 cells were seeded in 96-well plates at a density of 3000/well for 24 h, and treated with different concentrations of quercetin (0–200 µM) for 72 h. Cells were then incubated with MTT reagent (2 mg/mL, 50 µL) for 4 h. The effect of quercetin on cell proliferation was measured by incubating cells with 50 µM quercetin at different time points. The fraction of living cells was measured using an enzyme-linked immunosorbent assay plate reader.
as the optical density at 540 nm. IC\textsubscript {50} values were calculated using SigmaPlot software.

**Flow Cytometry** FACS analysis was employed to determine the fraction of apoptotic cells. Each NSCLC cell line was treated with quercetin (50 µM) for various periods of time (1, 2, or 4 d). Cell cycle fractions were assessed using 1×10\textsuperscript{6} cells, which were fixed in 80% ethanol, stained with PI solution, and analyzed in a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, U.S.A.). Another apoptosis assay was conducted by dual-laser flow cytometry.\textsuperscript{27} Following quercetin treatment, cells were stained with Hoechst (1 µg/mL) and then with 7-AAD (1 µg/mL). The co-stained cells were then analyzed.

**Western Blot Analysis** Each NSCLC cell line was treated with 50 µM quercetin for 2 d and lysed in lysis buffer (0.2 M ethylenediaminetetraacetic acid, 0.05 M Tris–Cl (pH 8.0), 5% Triton X-100) for 30 min on ice. For validation assays, H460 cells were treated with two different concentrations of quercetin (25, 50 µM). The lysates were resolved by electrophoresis on 8–12% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with the following primary antibodies: anti-caspase-3 (235412; CalBiochem, San Diego, CA, U.S.A.), anti-caspase-8 (AM46; Oncogene, Cambridge, MA, U.S.A.), anti-poly(ADP-ribose) polymerase (PARP) (sc-7150; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-NF-κB p50 (sc-7178), anti-IκBα (sc-1643), anti-FAS (sc-715), anti-growth arrest and DNA-damage inducible 45 (GADD45) (sc-797), anti-tumor necrosis factor-related apoptosis-inducing ligand receptor (TRAILR) (sc-11638), anti-p21\textsuperscript{Cip1} (sc-6246), and anti-β-actin (sc-4778). The blots were then incubated with a horse-radish peroxidase-conjugated mouse (sc-2005) or rabbit (sc-2004) immunoglobulin G

<table>
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<th>Name</th>
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Fig. 1. Cytotoxic Effect of Quercetin on the Growth of NSCLC Cells

(A) Cytotoxicity of quercetin. IC\textsubscript {50} values in A549 (■), H1299 (●), and H460 (○) cells were determined by MTT assays in the presence of various concentrations of quercetin (1–200 µM). (B–D) Effects of quercetin on cell proliferation. A549 (B), H1299 (C), and H460 (D) cells were incubated with 50 µM quercetin or DMSO for the indicated periods of time. Cell growth was monitored by MTT assay. Data are the mean±standard deviation (S.D.) of three independent experiments. Asterisks indicate significant differences between DMSO- and quercetin-treated cells (*p<0.05).
(IgG) secondary antibody. Protein bands were detected using the Enhanced WEST-ZOL System (iNtRON Biotechnology, SungNam, South Korea).

Hoechst Staining  H460 cells were seeded on coverslips and then treated with quercetin (0–100 µM) for 2 d. After two washes in phosphate-buffered saline (PBS), the cells were stained with Hoechst staining solution for 5 min at room temperature. Coverslips were washed, dried, and mounted on microscopic slides. Apoptotic nuclear morphology was imaged with a fluorescence microscope (model DM2500; Leica Microsystems, Wetzlar, Germany).

Trypan Blue Exclusion  H460 cells were treated with quercetin (0–100 µM) for 2 d and collected by centrifugation. After washing in PBS, cells were stained with 0.4% trypan blue solution at room temperature for 3 min, and populations were then counted using a hemocytometer and a light microscope. One thousand cells were observed and the percentages of unstained (viable) and stained (nonviable) cells were determined.

**DNA Fragmentation Analysis**  H460 cells were treated with quercetin (0–100 µM) for 2 d and collected by centrifugation. After washing in PBS, cells were lysed as described above and extracted with phenol. After ethanol precipitation, DNA was electrophoretically separated on a 1.5% agarose gel containing ethidium bromide (1 µg/mL) and visualized using the Chemi doc XRS system (Bio-Rad, Hercules, CA, U.S.A.).

**Microarray and Data Analysis**  Total RNA was extracted from H460 cells treated with quercetin (50 µM) for 2 d. Qualified RNA samples with an RNA integrity number of >9 were used for further analysis in a two-color microarray experiment using human 44k 4plex arrays (Agilent, Santa Clara, CA, U.S.A.), according to the manufacturer’s instructions. Equal amounts of total RNA were amplified, labeled, hybridized, washed, and scanned. The locally weighted linear regression curve fit and dye-swap normalization methods were applied to the ratio (Cy5/Cy3) of the signal intensities generated in the microarrays. Results were filtered, and the cutoff was set at $p<0.05$. Genes exhibiting significant differences in expression...
Results and Discussion

Cytotoxic Effect of Quercetin on Lung Cancer Cells

To compare the inhibitory effects of quercetin on the growth of three lung cancer cells, we measured IC₅₀ values following MTT assays. The IC₅₀ values for quercetin were 106.3 (A549 cells), 58.3 (H1299 cells), and 92.2 (H460 cells) (Fig. 1A). This effect of quercetin appears to be independent of p53 status because both A549 and H460 cells harbor wild-type p53, while H1299 cells are p53-deficient. The effect of quercetin was substantiated by additional MTT assays at various time points (Figs. 1B–D). Cells were treated with DMSO (as a control; gray line) or quercetin (50 µM; black line) for 5 d. No growth was observed in quercetin-treated cells, unlike in control cells.

Apoptotic Effect of Quercetin on Lung Cancer Cells

Two sequential FACS analyses were conducted to determine whether the growth inhibitory effect of quercetin was due to the induction of apoptosis, a form of programmed cell death. Cells were treated with quercetin (50 µM) for 2 d and then subjected to flow cytometric analysis followed by PI staining. The sub-G₁ fraction (representing apoptotic cells) was increased in quercetin-treated H1299 and H460 cells (H1299-Q) (47%) and H460 cells (H460-Q) (88%). A second FACS analysis using Hoechst and 7-AAD was performed to distinguish live cells, and early and late apoptotic cells. The early and late apoptotic populations greatly increased in quercetin-treated H1299 and H460 cells compared to A549 cells (Fig. 2B). The apoptotic potential of quercetin in the three NSCLC cell lines was compared by Western blotting of three apoptosis-related proteins: caspase-3, caspase-8, and PARP. As shown in Fig. 2C, quercetin significantly increased cleavage of caspase-3 and caspase-8 in H1299 and H460 cells, but not in A549 cells. A similar pattern for PARP cleavage was observed. Taken together, these data suggest that quercetin induces apoptosis in the three NSCLC cell lines, and that H460 cells were slightly more sensitive than H1299 cells.

Quercetin-induced apoptosis in H460 cells was further confirmed by three independent experiments. First, DNA-binding Hoechst dye was used to examine the nuclear morphology of NF-κB mutant binding element (Bcl2-NF-κB mt-Luc), and SV-40-driven β-galactosidase expression vectors using LipofectAMINE (Invitrogen). H460 cells were co-transfected with the NF-κB expression vector as a positive control. After transfection overnight, the cells were treated with DMSO (control) or quercetin (50 µM). Luciferase activity was measured as described previously.²⁹

Plasmids and Cloning

NF-κB (p50) cDNA was constructed according to standard methods and verified by sequencing. The p50 cDNA was amplified by PCR and subcloned into Flag (2×)-tagged pcDNA3 vectors for over-expression, and pSilencer 2.1-U6 Hygro (Applied Biosystems/Amnbion, Austin, TX, U.S.A.) for depletion of endogenous NF-κB (p50). DNA sequence used for small hairpin (sh) RNA (shp50) construction was 5’-GATCCG CGA GAGTTT ACA TCT GAT GAT CTC AA GA G GAT CAT CAG ATG TAA CTG GTT TTG GAA A-3’.

RESULTS AND DISCUSSION

RNA Extraction and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from H460 cells treated with quercetin (50 µM) for 2 d, and 2 µg of total RNA was reverse-transcribed using MMLV reverse transcriptase and random oligo(dT) primers (Invitrogen). Real-Time PCR was conducted using primer pairs (Table 1), iQ™ SYBR Green Supermix, and the iCycler CFX96 Real-Time PCR Detection System (Bio-Rad). All gene expression levels in each well were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Fold-expression was defined as the fold-increase relative to controls.

Fluorescence Microscopy

H460 cells seeded on cover slides were treated with DMSO (control) or quercetin (50 µM) for 2 d. The cells were fixed and permeabilized as described previously.²⁸ After washing, the cells were incubated with anti-NF-κB p50 subunit (sc-7178) and anti-IκBα (sc-1643) antibodies in 3% bovine serum albumin blocking solution for 1 h and then incubated with goat anti-rabbit IgG Texas Red (sc-2010). After washing, the cells were visualized under a confocal microscope (model TCS SPS; Leica Microsystems). Hoechst (Sigma) was used to visualize chromosomal DNA.

Luciferase Reporter Gene Assays

H460 cells were seeded in 12-well plates and co-transfected with luciferase reporter genes, the NF-κB-responsive Bcl2 gene reporter with the NF-κB binding element (Bcl2-NF-κB wt-Luc) or the NF-κB mutant binding element (Bcl2-NF-κB mt-Luc), and SV-40-driven β-galactosidase expression vectors using LipofectAMINE (Invitrogen). H460 cells were co-transfected with the NF-κB expression vector as a positive control. After transfection overnight, the cells were treated with DMSO (control) or quercetin (50 µM). Luciferase activity was measured as described previously.²⁸

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dead cells. Quercetin-treated cells displayed typical morphological features of apoptotic cells, with condensed and fragmented nuclei (Fig. 2d). Second, a DNA fragmentation assay was conducted. DNA laddering, a hallmark of apoptosis, was detected in cells treated with higher concentrations of quercetin (50, 100 µm) (Fig. 2E). Finally, the trypan blue exclusion test was performed to determine the proportion of viable cells. Quercetin reduced the percentage of unstained (viable) cells in a dose-dependent manner (Fig. 2F). Overall, these data support the conclusion that quercetin is a potent inducer of apoptosis in H460 cells.

Microarray Analysis of Quercetin-Regulated Genes in H460 Cells

To determine the molecular mechanism underlying quercetin-induced apoptosis in H460 cells, we performed microarray assays using cDNA from H460 cells treated with quercetin or DMSO (control) for 2 d. We identified 6620 genes that were more than twofold differentially expressed: 3150 were upregulated and 3470 down-regulated. Gene sets (>1.5-fold difference) associated with apoptosis and the cell cycle were used in a cluster analysis. As shown in Fig. 3, quercetin upregulated genes associated with death pathways, including death receptors (TRAILR, FAS, TNFR1), the c-Jun N-terminal kinase (JNK) pathway (MEKK1, M KK4, JNK), the interleukin-1 receptor pathway (IL1, IL1R, IRAK), the caspase cascade (caspase-10, DFF45), and the NF-κB pathway (IκB), while it down-regulated genes involved in cell survival (NF-κB, IKK, AKT). In addition, quercetin increased the expression of GADD45, which is associated with cell cycle arrest, but decreased the expression of genes related to cell proliferation (SCF, SKP2, CDKs, cyclins).

Validation of Microarray Data

Some of the quercetin-regulated genes were validated by quantitative RT-PCR and Western blotting analysis. Quantitative RT-PCR assays indicated that quercetin augmented the expression of apoptosis-related genes (FAS, TRAILR), the NF-κB repressor IκB, and genes involved in cell cycle arrest (p21Cip1, GADD45) (Fig. 4A). In contrast, quercetin repressed the expression of NF-κB, a transcription factor required for cancer cell survival. The protein expression of these regulated genes was subsequently monitored by Western blotting (Fig. 4B). Overall, the Western blotting data were fairly consistent with the microarray data, with little difference in magnitude.

Quercetin treatment decreased the expression of p50, a subunit of NF-κB, but increased the expression of its inhibitor, IκB. To determine the biological significance of this opposite regulation, we first performed immunofluorescence microsco-
Following quercetin treatment, the nuclear level of NF-κB (p50) decreased greatly, whereas IκBα expression increased slightly (Fig. 4C). Then, luciferase reporter gene assays were employed. Quercetin significantly inhibited luciferase activity in H460 cells in transfection experiments using an NF-κB-responsive Bcl2 promoter fused to a luciferase reporter (Fig. 4D). In contrast, a mutant reporter carrying a mutation in the NF-κB binding site was unaffected by quercetin and NF-κB.

To determine whether the quercetin effect on cell fate is closely dependent on the NF-κB expression, regardless of IκBα, we measured the effect of NF-κB on the quercetin-regulated cell growth and cell death in three NSCLS cells. With finding on down-regulation of NF-κB (p50) by quercetin, we observed that the proliferation potential of quercetin-treated and NF-κB (p50)-transfected cells (Q+F-p50) is higher than that of untransfected- and quercetin-treated cells (Q+F-emp). This effect was observed in all three lung cancer cell lines, A549, H1299, and H460 cells (Fig. 5). Quercetin-induced cell death (shown by increased subG1 population) was also rescued by NF-κB (p50) overexpression in all three NSCLC cells (Supplementary Fig. 1). The role of NF-κB in cell survival was demonstrated by similar assays using NF-κB (p50)-depleted cells. These data support that quercetin induces apoptosis/cell death by inhibiting NF-κB signaling through the down-regulation of NF-κB (p50). Based on independent role of NF-κB, we expect that quercetin-induced up-regulation of IκB could be addictive in mediating cell death in NSCLS cells.

In this study, we investigated the anticancer effect of quercetin and the underlying molecular mechanism in NSCLC cells. Our cytotoxicity assay data indicate that quercetin strongly inhibited the growth of all three tested NSCLC cell lines (A549 [p53 wt], H1299 [p53 null], and H460 [p53 wt]), suggesting that quercetin-triggered cytotoxicity is independent of p53. However, in terms of apoptosis, these cells responded differently to quercetin: only H460 and H1299 cells were affected, with H460 cells being slightly more sensitive. Further microarray analysis using quercetin-treated H460 cells and cluster analyses revealed that quercetin regulated numerous genes associated with apoptosis and the cell cycle. As shown in Fig. 5A, quercetin upregulated genes associated with death pathways, including death receptors, the JNK pathway, the interleukin-1 receptor pathway, the caspase cascade, and NF-κB inhibition, but down-regulated genes involved in cell survival. In addition, quercetin increased the expression of GADD45, which is associated with cell cycle arrest, but decreased the expression of genes related to cell proliferation (Fig. 5B). For selected quercetin-regulated genes (FAS, TRAILR, IκBα, NF-κB, p21CIP1, GADD45), we demonstrated differential regulation of mRNA and protein expression. Notably, our attention was drawn to the fact that NF-κB was down-regulated and IκBα upregulated because they are involved in cell survival and NF-κB inhibition, respectively. Next, we explored the

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**Fig. 5. Effect of NF-κB Expression on Quercetin-Induced Cytotoxicity in NSCLC Cells**

(A) Expression of NF-κB p50 subunit in A549, H1299, and H460 under various conditions. (B–C) Effect of NF-κB expression level on proliferation of A549 (B), H1299 (C), and H460 (D) cells. Each cell line was incubated with 50 µM quercetin (Q) or DMSO (D) under overexpression (F-p50) or depletion condition (shp50) of NF-κB p50 for the indicated times. Flag tagged-empty vector (FE) and shRNA for Luciferase (shLuc) were used as controls. Cell growth was monitored by MTT assay. Data are the mean±standard deviation (S.D.) of three independent experiments. Asterisks indicate significant differences between DMSO-treated cells (D+FE) and other treated cells (*p<0.05).
biological significance of this opposite regulation and concluded that quercetin mediates growth inhibition in H460 cells by suppressing the cell survival factor NF-κB and increasing the expression of death receptors and cell cycle inhibitors. Although its efficacy in mouse lung cancer models remains to be determined, our findings support the conclusion that quercetin may potentially be used to prevent and treat human NSCLC.

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REFERENCES

15) Chirumbolo S. The role of quercetin, flavonols and flavones in modulating inflammatory cell function. Inflamm. Allergy Drug Targets,


30) Dyson HJ, Komives EA. Role of disorder in IκB-NFκB interaction. JUBMB Life, 64, 499–505 (2012).