Survivin and p53 Modulate Quercetin-induced Cell Growth Inhibition and Apoptosis in Human Lung Carcinoma Cells*

Received for publication, July 15, 2004, and in revised form, September 23, 2004
Published, JBC Papers in Press, September 29, 2004, DOI 10.1074/jbc.M407985200

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Quercetin, a ubiquitous bioactive plant flavonoid, has been shown to inhibit the proliferation of cancer cells. However, the regulation of survivin and p53 on the quercetin-induced cell growth inhibition and apoptosis in cancer cells remains unclear. In this study, we investigated the roles of survivin and p53 in the quercetin-treated human lung carcinoma cells. Quercetin (20–80 μM for 24 h) induced the cytotoxicity and apoptosis in both A549 and H1299 lung carcinoma cells in a concentration-dependent manner. Additionally, quercetin inhibited the cell growth, increased the fractions of G2/M phase, and raised the levels of cyclin B1 and phospho-cdc2 (threonine 161) proteins. Moreover, quercetin induced abnormal chromosome segregation in H1299 cells. The survivin proteins were highly expressed in mitotic phase and were located on the midbody of cytokinesis; however, the survivin proteins were increased and concentrated on the nuclei following quercetin treatment in the lung carcinoma cells. Transfection of a survivin antisense oligodeoxynucleotide enhanced the quercetin-induced cell growth inhibition and cytotoxicity. Subsequently, quercetin increased the levels of total p53 (DO-1), phospho-p53 (serine 15), and p21 proteins, which were translocated to the nuclei in A549 cells. Treatment with a specific p53 inhibitor, pifithrin-α, or transfection of a p53 antisense oligodeoxynucleotide enhanced the cytotoxicity of the quercetin-treated cells. Furthermore, transfection of a small interfering RNA of p21 enhanced the quercetin-induced cell death in A549 cells. Together, our results suggest that survivin can reduce the cell growth inhibition and apoptosis, and p53 elevates the p21 level, which may attenuate the cell death in the quercetin-treated human lung carcinoma cells.

Survivin is expressed in human cancer cells, but it is undetectable in most normal adult cells (1–3). It has been shown that survivin may exhibit anti-apoptotic effect and inhibit the activity of caspases in cancer cells (3–6). The survivin activity resulted from the phosphorylation of threonine 34 by the mitotic kinase complex cdc2/cyclin B1 (7, 8). Survivin has been demonstrated to promote mitotic progression (2, 7, 8) and cytokinesis (9–11) in cancer cells. Moreover, it has been proposed that survivin may serve as a radio- and chemoresistance factor (8, 12). Treatments with adriamycin and taxol increase survivin expression in cancer cells (8). However, a semisynthetic flavonoid, flavopiridol, suppresses the survivin phosphorylation on threonine 34 and enhances cancer cell apoptosis induced by anticancer agents, e.g. adriamycin and UVB irradiation (8).

p53 is a cellular gatekeeper for the cell growth and division (13–15). It has been shown that p53 can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells (14, 15). The p53 downstream effectors include p21, which participates in cell cycle arrest, and bax, which triggers apoptosis (13, 14). Inhibition of p53 function may cause the decrease of DNA repair and the increase of genomic instability (16, 17). Also, the p53 protein mediates the G1 and G2/M checkpoints, which trigger the p21 to participate in cell cycle arrests (18). In addition, p21 can play a protective role on survival signal against apoptosis (19–21). The diverse phosphorylation sites of p53 have been indicated to play important roles in the regulation of many cellular responses (22–24). Moreover, it has been proposed that the phosphorylation of p53 at serine 15 is an important target for p53 activation (25) and stabilization (22, 26).

The antitumor effects of plant flavonoids have been reported to induce cell growth inhibition and apoptosis in a variety of cancer cells (27–29). Quercetin, a ubiquitous bioactive flavonoid, can inhibit the proliferation of cancer cells (30–32). It has been shown that quercetin treatment caused cell cycle arrests such as G2/M arrest or G1 arrest in different cell types (31–34). Moreover, quercetin-mediated apoptosis may result from the induction of stress proteins, disruption of microtubules and mitochondrial, release of cytochrome c, and activation of caspases (32, 35–37). However, the roles of survivin and p53 proteins in the quercetin-induced cell growth inhibition and apoptosis in cancer cells are still not clear.

In this study, we investigated the expression of survivin, p53, and p21 proteins in quercetin-induced cell growth inhibition and apoptosis of the human lung cancer cells. We further studied the effects of survivin, p53, and p21 gene knockdowns by using the antisense oligodeoxynucleotides of survivin, p53, and a small interfering RNA (siRNA)3 of p21, respectively, in the quercetin-treated lung carcinoma cells. The enhanced survivin expression may act to reduce cell growth inhibition and apoptosis, and p53-mediated elevation of the p21 level may moderate cell death in the quercetin-treated human lung carcinoma cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies—**Hoechst 33258, pifithrin-α, propidium iodide, survivin, and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazo-

* This work was supported by Grant NSC 93-2320-B-320-014 from the National Science Council, Taiwan and Tzu Chi University Grant TCMRC 92009. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; F-actin, actin filament.
FIG. 1. Effects of quercetin on cytotoxicity and apoptosis in human lung carcinoma cells. A, the cells were treated with 0–80 µM quercetin for 24 h. The cell survival was measured by MTT assay. Results were obtained from 3–5 experiments, and the bars represent the mean ± S.E. B, the cells were treated with 40 µM quercetin for 24 h in A549 cells. The nuclei were stained with propidium iodide, which displayed red fluorescence (arrows). Annexin V-FITC displayed green fluorescence (stars). C, the A549 cells were treated with 60 µM quercetin for 24 h. The cells

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FIG. 2. Effects of quercetin on cell growth and cell cycle progression in human lung carcinoma cells. A, the cells were plated at a density of $1 \times 10^6$ cells/100 Petri dish for 18 h. Then the cells were treated with 0–80 μM quercetin for 24 h. After drug treatment, the cells were washed twice with PBS and incubated for various times before they were counted by a hemocytometer. Results were obtained from 5–6 experiments, and the bars represent mean ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**) indicate the difference between untreated and quercetin-treated samples. B, the cells were treated with 0–60 μM quercetin for 24 h. After treatment, the cells were trypsinized and then subjected to flow cytometry analyses. The data represented the average values from three experiments. C, the cells were exposed to 0–60 μM quercetin for 24 h. The total protein extracts were prepared for Western blot analysis using anti-cdc2, anti-cyclin B1, anti-cyclin D1, anti-phospho-cdc2 (threonine 161), and anti-ERK-2 antibodies.

were incubated with rabbit anti-apoptosis inducing factor antibody and then incubated with goat anti-rabbit Cy5. The apoptosis inducing factor protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei were stained with Hoechst 33258, which displayed blue fluorescence. Arrows indicate the apoptotic nuclei. D, the H1299 cells were treated with 60 μM quercetin for 24 h. The F-actin was stained with BODIPY FL phallacidin, which displayed green fluorescence. Arrows indicate the apoptotic nuclei. E, the percentage of apoptosis was scored from the apoptotic nuclei. Results were obtained from 4–5 experiments, and the bars represent mean ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**) indicate the difference between untreated and quercetin-treated samples.
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Fig. 3. The expression and location of survivin proteins in a variety of human cancer cells. A, the total protein extracts were prepared for Western blot analysis using anti-survivin and anti-ERK-2 antibodies. B, the cells were incubated with rabbit anti-survivin antibody and then incubated with goat anti-rabbit Cy5. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei were stained with Hoechst 33258, which displayed blue fluorescence. The F-actin was stained with BODIPY FL phallacidin, which displayed green fluorescence. Arrows indicate the location of survivin proteins in the midbody of cytokinesis.

Cell Cycle Analysis—The cells were plated at a density of 1 × 10⁶ cells per 100 Petri dish in complete RPMI 1640 medium for 16–18 h. Then the cells were treated with 0–80 μM quercetin for 24 h in serum-free RPMI 1640 medium. At the end of treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20 °C. After centrifugation, the cell pellets were treated with 4 μg/ml propidium iodide solution containing 100 μg/ml RNase and 1% Triton X-100 for 30 min. Subsequently, the samples were analyzed in a FACScan light-scattering system (BD Biosciences) using CellQuest software. The percentage of cell cycle phases was analysis by a ModFit LT software (Version 2.0, BD Biosciences).

Cell Growth Assay—The cells were plated at a density of 1 × 10⁶ cells per 100 Petri dish in complete RPMI 1640 medium for 16–18 h. Then the cells were treated with 0–80 μM quercetin for 24 h in serum-free RPMI 1640 medium. After drug treatment, the cells were washed twice with PBS and re-cultured in complete RPMI 1640 medium. Subsequently, the cells were incubated for various times before they were counted by a hemocytometer.

Indirect Immunofluorescence and Confocal Microscopy—The cells were cultured on coverslips, which were kept in a 96 well Petri dish for 16–20 h before treatment. After treatment with or without 60 μM quercetin for 24 h, the cells were washed with isotonic PBS (pH 7.4) and then fixed in 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Then the coverslips were washed three times with PBS, and nonspecific binding sites were blocked in PBS containing 10% normal goat serum, 0.3% Triton X-100 for 1 h. The cells were incubated with rabbit anti-survivin antibody (1:250) in PBS containing 0.3% Triton X-100 and 10% normal goat serum overnight at 4 °C and washed three times with 0.3% Triton X-100 in PBS. Then the cells were incubated with goat anti-rabbit Cy5 (1:250) in PBS containing 0.3% Triton X-100 and 10% normal goat serum for 2–3 h at 37 °C and washed three times with 0.3% Triton X-100 in PBS. The nuclei were stained with 2.5 μg/ml Hoechst 33258 for 10 min. The number of apoptotic nuclei was counted by a hemocytometer under a fluorescence microscope. A total of 200 cells were examined for the calculation of apoptotic percentage in each treatment.

Apoptosis Assay—The adherent cells were cultured on coverslips. After exposure to quercetin, the cells were washed twice with phosphate-buffered saline (PBS) and were re-cultured in complete RPMI 1640 medium (containing 10% fetal bovine serum) for 2 days. Subsequently, the medium was replaced, and the cells were incubated with 0.5 mg/ml of MTT in complete RPMI 1640 medium for 4 h. The surviving cells converted MTT to formazan that generates a blue-purple color when dissolved in dimethyl sulfoxide. The intensity was measured at 545 nm for the calculation of apoptotic percentage in each treatment.

Cytotoxicity Assay—The cells were plated in 96-well plates at a density of 1 × 10⁴ cells/well for 16–20 h. Then the cells were treated with 0–80 μM quercetin for 24 h in serum-free RPMI 1640 medium. After drug treatment, the cells were washed twice with PBS and re-cultured in complete RPMI 1640 medium. After treatment with or without 60 μM quercetin for 24 h, the cells were washed with isotonic PBS (pH 7.4) and then fixed in 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Then the coverslips were washed three times with PBS, and nonspecific binding sites were blocked in PBS containing 10% normal goat serum, 0.3% Triton X-100 for 1 h. The cells were incubated with rabbit anti-survivin antibody (1:250) in PBS containing 0.3% Triton X-100 and 10% normal goat serum overnight at 4 °C and washed three times with 0.3% Triton X-100 in PBS. Then the cells were incubated with goat anti-rabbit Cy5 (1:250) in PBS containing 0.3% Triton X-100 and 10% normal goat serum for 2–3 h at 37 °C and washed three times with 0.3% Triton X-100 in PBS. The nuclei were stained with 2.5 μg/ml Hoechst 33258 for 10 min. The number of apoptotic nuclei was counted by a hemocytometer under a fluorescence microscope. A total of 200 cells were examined for the calculation of apoptotic percentage in each treatment.

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Apoptosis Assay—The adherent cells were cultured on coverslips. After exposure to quercetin, the cells were washed twice with isotonic PBS (pH 7.4). The cells were stained with annexin V-FITC (Strong Biotech, Taipei, Taiwan) in binding buffer containing propidium iodide for 30 min in the dark. Then the cells were washed three times with isotonic PBS and analyzed using a fluorescence microscope.

To score the percentage of apoptosis, the adherent cells were cultured on coverslips. After drug treatment, the cells were washed with isotonic PBS (pH 7.4) and then fixed in 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Then the nuclei were stained with 2.5 μg/ml Hoechst 33258 for 10 min. The number of apoptotic nuclei was counted by a hemocytometer under a fluorescence microscope. A total of 200 cells were examined for the calculation of apoptotic percentage in each treatment.
Western Blot Analysis—Western blot analyses of cyclin B1, cyclin D1, cdc2, phospho-cdc2 (threonine 161), survivin, p21, p53 (DO-1), phospho-p53 (serine 15), and ERK-2 were performed using specific antibodies. After treatment, the cells were lysed in ice-cold cell extract buffer (pH 7.6) containing 0.5 mM dithiothreitol, 0.2 mM EDTA, 20 mM HEPES, 2.5 mM MgCl₂, 5 mM NaCl, 0.1 mM Na₃VO₄, 0 mM NaF, and 0.1% Triton X-100. The protease inhibitors including 1/1000 g/ml aprotinin, 0.5/1000 g/ml leupeptin, and 100/1000 g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride were added to the cell suspension. The protein concentrations were determined by the BCA protein assay kit (Pierce). Equal amounts of proteins (20–80 µg/well) were subjected to electrophoresis using 10–12% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoretic transfer of proteins onto polyvinylidene difluoride membranes, the proteins were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Inc.). Finally, the protein bands were visualized using the enhanced chemiluminescence detection system (PerkinElmer Life Sciences).

Transfection—The antisense oligodeoxynucleotides were synthesized by Medclub Scientific Co. (Taipei, Taiwan) in the form of phosphorothioate oligodeoxynucleotides. The sequence of survivin antisense oligodeoxynucleotide was 5'-CCCAGCCTTCCAGCTCTTG-3' (41), p53 antisense oligodeoxynucleotide was 5'-CCCTGCTCCCCCCTGGCTCC-3', and the control oligodeoxynucleotide was 5'-GGAGCCAGGGG-GAGCAGGG-3'. These oligodeoxynucleotides were employed for transfections in A549 cells. The cells (5 × 10⁵ cells/p35 dish) were transfected with 100 nM of control or antisense oligodeoxynucleotides by using LipofectAMINE²* 2000 (Invitrogen) in 1 ml of serum-free medium for 5 h at 37 °C in a CO₂ incubator according to the manufacturer’s recommendations. Then, 1 ml of Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum was added without removing the transfection mixture, and incubation proceeded for an additional 24 h. In addition, a p21 siRNA (Santa Cruz Biotechnology, Inc.) was utilized for the p21 gene knockdown in A549 cells according to the manufacturer’s recommendations. After transfection, the cells were subjected to cell growth or MTT assay as described above.

Statistical Analysis—Data were analyzed using Student’s t-test, and significant differences were found between values obtained in the population of cells treated with different conditions. A p value of < 0.05 was considered to be statistically significant in the experiments.

RESULTS
Quercetin Induces Cytotoxicity and Apoptosis in Human Lung Carcinoma Cells—The human lung cancer cell lines were treated with 0–80 µM quercetin for 24 h. As shown in Fig. 1A, quercetin induced cell death in a concentration-dependent manner, and ~70 and 30% of A549 and H1299 cells, respec-
tively, survived after exposure to 40 μM quercetin for 24 h. However, the LC50 values of quercetin toward cultured human normal lung fibroblasts were >120 μM (results not shown). We further assessed apoptosis from the cells that had been exposed to quercetin. Quercetin-treated cells were heavily stained with annexin V-FITC (early apoptotic cells) that displayed green color (Fig. 1B, lower right panel, stars) and increased the nuclear fragmentation and apoptotic bodies (Fig. 1C and Fig. 1D, arrows) in both A549 and H1299 cells. Quercetin partially induced the translocation of apoptosis inducing factor into the nuclei in A549 cells (Fig. 1C). Moreover, quercetin caused a significantly higher level of apoptosis inducing factor translocation into the nuclei of H1299 cells (data not shown). The late apoptotic cells were stained with annexin V-FITC and propidium iodide (Fig. 1B, lower right panel, arrows). In contrast, the control cells were not significantly stained with fluorescence microscope. As shown in Fig. 1E, the percentage of apoptosis was increased by quercetin (40–60 μM) in A549 cells in a concentration-dependent manner. Quercetin at 60 μM induced about 25% of apoptosis in H1299 cells (Fig. 1E).

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**FIG. 5.** Effects of quercetin on survivin expression and chromosome segregation in H1299 cells. A, H1299 cells were treated with 60 μM quercetin for 24 h. The cells were incubated with rabbit anti-survivin antibody and then incubated with goat anti-rabbit Cy5. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei were stained with Hoechst 33258, which displayed blue fluorescence. The F-actin was stained with BODIPY FL phallacidin, which displayed green fluorescence. B and C, the cells were treated with 60 μM quercetin for 24 h in H1299 cells. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei were stained with Hoechst 33258, which displayed blue fluorescence. Arrows indicate the location of survivin proteins on the midbody of cytokinesis.

**Quercetin and Cancer**

Quercetin induces cell growth inhibition and increases the fractions of G2/M phase in human lung carcinoma cells—To examine the effect of quercetin on tumor cell growth, A549 cells were plated at a density of 1 × 10^6 cells per p100 dish and were treated with 0–80 μM quercetin for 24 h. Cell numbers were counted using a hemocytometer. As shown in Fig. 2A, quercetin inhibited the cell growth in a concentration-dependent manner in A549 cells. Higher concentrations of quercetin (60 and 80 μM) almost completely inhibited the cell proliferation (Fig. 2A). To further determine the possible involvement of quercetin in the regulation of cell cycle, the effect of quercetin on lung carcinoma cells was analyzed by flow cytometry. Quercetin decreased the G1
fraction while increasing the G2/M fraction in both A549 and H1299 cells (Fig. 2B). Moreover, immunoblot analysis showed that quercetin increased the levels of cyclin B1 and phospho-cdc2 (threonine 161) proteins in both A549 and H1299 cells (Fig. 2C). However, quercetin did not significantly change the levels of cyclin D1 and cdc2 proteins in these cells (Fig. 2C). ERK-2 protein was used as an internal control in this study.

The Expression and Location of Survivin Protein in a Variety of Human Cancer Cells—To examine the expression of survivin in cancer cells, total cellular proteins were extracted and subjected to immunoblot analysis. As shown in Fig. 3A, the human cancer cell lines including lung cancer (A549 and H1299), colon cancer (RKO and SW480), cervical cancer (HeLa), and breast cancer (MCF-7) expressed the high levels of survivin proteins. To further study the location of survivin protein, the cancer cells were subjected to immunofluorescence staining and confocal microscopy. As shown in Fig. 3B, the intensity of red fluorescence (Cy5) exhibited by survivin was expressed in a variety of cancer cells. The survivin proteins were highly expressed in mitotic phase and concentrated on the midbody of cytokinesis of cancer cells (Fig. 3B and 4A, arrows).

Quercetin Increases the Levels of Total p53 (DO-1), Phospho-p53 (Serine 15), and p21 Proteins in A549 Cells—We have investigated the possible roles of p53 and p21 in the quercetin-induced cell growth inhibition and cytotoxicity induced by quercetin. A, the cells were plated at a density of 5 × 10⁵ cells/p60 Petri dish for 18 h. The survivin antisense oligodeoxynucleotide transfected-cells were exposed to 60 μM quercetin for 24 h in serum-free medium. Then the cells were washed twice with PBS and incubated for various times before they were counted by a hemocytometer. B, the cell survival was measured by MTT assay. Results were obtained from four experiments, and the bars represent ± S.E. p < 0.05 (*) and p < 0.01 (**) indicate the difference between the control and survivin antisense oligodeoxynucleotides-transfected-cells without quercetin treatment. p < 0.05 (*) and p < 0.01 (**) indicate the difference between the control and the survivin antisense oligodeoxynucleotides-transfected-cells after quercetin treatment.

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Quercetin Increases the Level of Survivin Proteins in Human Lung Carcinoma Cells—To investigate the effect of quercetin on the survivin protein expression, cells were treated with quercetin and subjected to immunofluorescence staining and immunoblot analysis. The survivin proteins were highly expressed in mitotic phase and concentrated on the midbody of cytokinesis in both A549 and H1299 cells (Fig. 4A and Fig. 5, arrows). Moreover, quercetin increased the level of survivin proteins in A549 cells (Fig. 4B) in a concentration-dependent manner. The red fluorescence (Cy5) intensity exhibited by survivin was significantly increased when exposed to 60 μM quercetin for 24 h in both A549 and H1299 cells (Fig. 4C and Fig. 5A). The increased survivin proteins were concentrated on the nuclei of these cells (Fig. 4C and Fig. 5A). In addition, quercetin induced abnormal chromosome segregation in H1299 cells (Fig. 5, B and C, lower panels). In this respect, quercetin induced a significantly higher degree of abnormal chromosome segregation in H1299 than in A549 cells (data not shown). The increased survivin proteins were co-localized with abnormal chromosomes in the quercetin-treated H1299 cells (Fig. 5, B and C, lower panels).

Survivin Antisense Oligodeoxynucleotide Enhances Quercetin-induced Cell Growth Inhibition and Cytotoxicity—A survivin antisense oligodeoxynucleotide was used for transfection in A549 cells to examine the effect of survivin on quercetin-induced cell growth inhibition and cell death. As shown in Fig. 6A, treatment with quercetin (60 μM for 24 h) or a transfection of survivin antisense oligodeoxynucleotide inhibited the cell growth in A549 cells. Moreover, the inhibition of cell growth caused by quercetin (60 μM for 24 h) was significantly enhanced by transfection with the survivin antisense oligodeoxynucleotides (Fig. 6A). To further investigate the role of survivin in quercetin-induced cytotoxicity, the survivin antisense oligodeoxynucleotide-transfected cells were treated with or without quercetin (60 μM for 24 h), and the percentage of cell survival was estimated by MTT assay. As shown in Fig. 6B, treatment with quercetin or transfection of a survivin antisense oligodeoxynucleotide induced cell death in A549 cells. Subsequently, the cytotoxicity caused by quercetin was significantly enhanced by transfection with the survivin antisense oligodeoxynucleotides (Fig. 6B).

FIG. 6. Effects of a survivin antisense oligodeoxynucleotide on the cell growth inhibition and cytotoxicity induced by quercetin. A, the cells were plated at a density of 5 × 10⁵ cells/p60 Petri dish for 18 h. The survivin antisense oligodeoxynucleotide transfected-cells were exposed to 60 μM quercetin for 24 h in serum-free medium. Then the cells were washed twice with PBS and incubated for various times before they were counted by a hemocytometer. B, the cell survival was measured by MTT assay. Results were obtained from four experiments, and the bars represent ± S.E. p < 0.05 (*) and p < 0.01 (**) indicate the difference between the control and the survivin antisense oligodeoxynucleotides-transfected-cells without quercetin treatment. p < 0.05 (*) and p < 0.01 (**) indicate the difference between the control and the survivin antisense oligodeoxynucleotides-transfected-cells after quercetin treatment.
mediated cytotoxicity of lung carcinoma cells. The green fluorescence (FITC) intensity exhibited by p53 (DO-1) was significantly increased following exposure to 60 \mu M quercetin for 24 h in A549 cells (Fig. 7A, lower right panel). A549 cells were treated with quercetin, and the protein level of p53 (DO-1) was assayed by the immunoblot. As shown in Fig. 7B, treatment with quercetin at 20–60 \mu M for 24 h or at 40 \mu M for 4–16 h significantly increased the levels of p53 (DO-1) proteins. In addition, quercetin (60 \mu M for 24 h) increased the level of phospho-p53 (serine 15) proteins, which were concentrated on the nuclei in A549 cells (Fig. 7C, arrows). Also, the p53 downstream p21 protein was increased (Fig. 7D) and concentrated on the nuclei in the quercetin-treated A549 cells (Fig. 7E, arrows).

Pifithrin-\(\alpha\), p53 Antisense Oligodeoxynucleotide, and p21 siRNA Enhance Quercetin-induced Cytotoxicity in A549 Cells—A specific p53 inhibitor, pifithrin-\(\alpha\) (42), or a p53 antisense oligodeoxynucleotide was used to examine the effects of p53 on the levels of cell death and p21 protein in quercetin-treated A549 cells. As shown in Fig. 8A, pifithrin-\(\alpha\) (20 \mu M for 24 h), which alone was without effect on cell survival, significantly enhanced the cytotoxicity mediated by quercetin. Similarly, transfection of a p53 antisense oligodeoxynucleotide enhanced the quercetin-induced cell death in A549 cells (Fig. 8B). Interestingly, transfection of a p53 antisense oligodeoxynucleotide reduced the elevation of p21 protein expression in the quercetin-treated A549 cells (Fig. 8C). To further investigate
FIG. 8. Effects of a p53 inhibitor or a p53 antisense oligodeoxynucleotide on the cytotoxicity and the level of p21 proteins in quercetin-treated A549 cells. A, the A549 cells were treated with 60 μM quercetin alone or in combination with 20 μM pifithrin-α for 24 h. The cell survival was measured by MTT assay. Results were obtained from 4–6 experiments, and the bars represent ± S.E. **, p < 0.01 indicates the difference between quercetin alone and quercetin in combination with pifithrin-α. B, the control or p53 antisense oligodeoxynucleotide-transfected cells were exposed to 60 μM quercetin for 24 h. The cell survival was measured by MTT assay. C, the total protein extracts were prepared for Western blot analysis using anti-p21, anti-p53, and anti-ERK-2 antibodies. D, the control or p21 siRNA transfected-cells were exposed to 40 μM quercetin for 24 h. The cell survival was measured by MTT assay. Results were obtained from three experiments, and the bars represent ± S.E. *, p < 0.05 indicates the difference between the control and the transfection with p53 antisense oligodeoxynucleotides or p21 siRNA after quercetin treatment.
reduce the cell death and to maintain the genome stability in quercetin-treated lung carcinoma cells.

The protective role of p53 is dependent on its downstream gene, p21 (20). p21 can participate in survival signal against apoptosis (19, 46). We observed that quercetin increased the levels of total p53, phospho-p53 (serine 15), and p21 proteins in A549 cells. Moreover, the phospho-p53 (serine 15) and p21 proteins were found to translocate to the nuclei of quercetin-treated cells. Phosphorylation of serine 15 was a key target site of p53 for its activation and stabilization (25, 26). The phosphorylation of p53 (serine 15) may transmit a survival signal and may suppress apoptosis in response to several stimuli (47). Additionally, the p21 may rescue cells from an apoptotic state to survival condition (19, 21). It has been shown that phosphorylation of p53 (serine 15) increases the level of p21 proteins and induces the G_{S/M} cell cycle checkpoint (47). In this study, a specific p53 inhibitor, pifithrin-α, or a p53 antisense oligodeoxynucleotide significantly enhanced the cytotoxicity concomitant with the decrease of p21 protein expression in the quercetin-treated A549 cells. Furthermore, transfection of p21 siRNA enhanced the cell death in the quercetin-treated A549 cells. Accordingly, our results suggest that p53 elevates the p21 level, which may attenuate the quercetin-induced cell death in lung carcinoma cells. However, the bax protein has been shown to be a downstream protein of p53 for the induction of apoptosis (14, 48). Thus, the role of p53 regulates the bax expression in the quercetin-induced apoptosis that need further investigation.

In summary, we propose that the survivin expression can reduce the cell growth inhibition and apoptosis, and the p53-regulated p21 protein expression appears the reduced cell death and aberrant mitosis in lung carcinoma cells following quercetin treatment (Fig. 9). Survivin may support p21/pro-caspase 3 complex formation resulting in the suppression of cell death signaling (49). Nevertheless, quercetin can mediate the differential pathways for aberrant mitosis and apoptosis via the induction of stress proteins, disruption of microtubules and mitochondrial, release of cytochrome c, and activation of caspases (32, 35–37). Understanding the mechanisms by which survivin, p53, and p21 pathways modulate the quercetin-induced cell cycle growth inhibition and apoptosis of cancer cells may contribute to the development of novel therapeutic strategies that can control the apoptotic and anti-apoptotic balance in such disease states.

Acknowledgments—We thank Dr. Ted H. Chiu for careful reading of the manuscript. We also thank Dr. T. C. Tsou for providing the p53 antisense oligodeoxynucleotides.

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