Reactive Oxygen Species Production Is Involved in Quercetin-Induced Apoptosis in Human Hepatoma Cells

Yuh-Fang Chang, Chin-Wen Chi, and Jane-Jen Wang

Abstract: Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality in Asia. The aim of this study was to examine whether reactive oxygen species production is involved in quercetin-induced apoptosis in human HCC cell lines. Quercetin inhibited the growth of hepatoma cells in dose and time dependent manners. Quercetin treatment of hepatoma cells resulted in changes of cell cycle progression. The G0/G1 phase was decreased and S phase was increased in HA22T/VGH cells after treatment with quercetin. The levels of apoptotic sub-G0/G1, reactive oxygen species and annexin V were increased prior to cell death and concurrent with lipid peroxidation in two human hepatoma cells after treatment with quercetin. Quercetin also enhanced the apoptotic effect of the chemotherapeutic agent, paclitaxel, in HA22T/VGH cells. Quercetin has therapeutic potential as an anti-cancer drug. These results provide basis for further study into the potential use of quercetin in combination with paclitaxel for treatment of hepatoma.

Quercetin, a bioflavonoid widely distributed in plants and fruits, has been found to have many biological activities, including anti- and pro-oxidant effects. Quercetin has been reported to induce elevation of glutathione (GSH) and γ-glutamylcysteine synthetase in human hepatoma cell line HepG2 (1). In addition, it was found that quercetin induced apoptosis in leukemia and pancreatic cancer cells (2,3). The quercetin-induced apoptosis in G1-S phase of tumor cells was dose dependent and heat shock protein HSP 70 appeared to be a mediator (4). Previously we have found that quercetin was effective in inhibiting the growth of murine hepatoma (5). The detailed mechanism of quercetin induced growth inhibition of hepatoma cells is not clear.

The defective apoptotic pathways play a pivotal role in the tumorigenesis. Effective chemotherapeutic agents, therefore, may prove to be those that promote both growth arrest and apoptosis. Common pathways of apoptosis implicate critical events like changes in cellular generation of reactive oxygen species (ROS), activation of caspases as well as increased binding of annexin V. ROS plays an important role in cell death by apoptosis or by necrosis (6). Mitochondrial respiration is the major biochemical pathway by which O2− is produced in the cells during oxidative phosphorylation for ATP generation. O2− is the major free radical species produced during normal aerobic metabolism and serves as a precursor for the formation of other ROS (7). An excessive accumulation of cellular O2− is cytotoxic and has been well-characterized as an inducer of apoptosis in a variety of cell types (8,9). Quercetin acts as an antioxidant, it was also shown that in high concentrations, they can generate ROS by autoxidation and redox-cycling (10–12). Although quercetin has been investigated for many years, quercetin induced pro-oxidant activity in hepatoma cells is not known. We hypothesize that the ROS production is involved in quercetin-induced anti-hepatoma effects.

Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality in Asia, and the only curative therapy is surgery. However, surgical treatment has limitations for patients with metastatic tumors. Searching for effective chemotherapeutic agents is important to increase the survival of patients with advanced or recurrent HCC after surgical treatment. Paclitaxel, a microtubules interfering agent, is one of the most active chemotherapeutic agents against a wide panel of solid tumors (13,14). It has been reported that paclitaxel can induce the formation of ROS and alter mitochondrial membrane permeability (15). The ROS production is involved in paclitaxel-induced apoptosis in hepatoma cells (16). Although the clinical trial of paclitaxel for patients with HCC showed only a limited response (17), whether combination therapy using quercetin and paclitaxel will be effective for patients with HCC is not known. The objective of this study was to examine the cytotoxic and antitumor effects of quercetin on two human HCC cell lines is relation to ROS and to explore the potential of using quercetin and paclitaxel for increasing cytotoxic response in tumor cells.

Y.-F. Chang and C.-W. Chi are affiliated with the Departments of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217, Taiwan. C.-W. Chi is also affiliated with the Institute of Pharmacology, National Yang-Ming University, Taipei 11217, Taiwan. J.-J. Wang is affiliated with the National Taipei College of Nursing, Taipei 11219, Taiwan.
Materials and Methods

Cell Culture
Two human hepatoma cell lines HA22T/VGH (established by Dr. Hu, Veteran General Hospital-Taipei) and HepG2 (ATCC: HB-8065) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% gentamycin (GIBCO). Cells were seeded at 5 × 10^4 cells per 25 cm^2 tissue flask in the growth medium and kept in a humidified CO_2 incubator at 37°C with 95% air and 5% CO_2.

Drug Treatment
Quercetin (Sigma, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO), and the final concentrations were 0, 40, 60, or 80 µM. The final concentration of DMSO was 0.1%. Cells were seeded and cultured for 24 h, and then replated with medium containing quercetin for another 24–72 h. To investigate the anticancer effect of quercetin in combination with chemotherapeutic agent, 0.1 µM paclitaxel (Sigma) was used. Cells were pretreated with quercetin for 24 h, washed twice with Hank’s buffer solution, and then re-plated with medium containing paclitaxel for another 48 h.

Cell Cycle Analysis
Cycle TEST™ PLUS DNA Reagent Kit (Beckton Dickinson, San Jose, CA) was used for staining of DNA. After washing the cells twice with sucrose buffer solution, the cell concentration was adjusted to 1 × 10^6/ml and 0.5 ml of cell suspension was centrifuged at 400 g for 5 min at room temperature (20–25°C). The cell pellet was added with 250 µl of solution A (trypsin buffer) and gently mixed. After incubation at room temperature for 10 min, 200 µl of solution B (trypsin inhibitor and RNase buffer) was added to each tube, gently mixed, and then incubated at room temperature for 10 min. This was followed with the addition of 200 µl of solution C [propidium iodide (PI) stain solution] and incubated for 10 min in the dark on ice (4°C). The cells were filtered through a 50-µm nylon mesh and used for flow cytometric analysis.

Flow Cytometry
Cells (20,000) were analyzed on a FASCalibur flow cytometer (Becton Dickinson) using an argon-ion laser (15 mW) with an incident beam at 488 nm. The red fluorescence (PI) was collected through a 585 nm filter. The data were analyzed by CellQuest, ver. 4.02 (Becton Dickinson) and ModFit, ver. 2.0 (Verity Software) softwares on a Macintosh computer.

Annexin V Binding Assay
To determine the extent of apoptosis, the annexin V-FITC kit (IMMUNOTECH, Marseille, France) was used. Cells in the amount of 1 × 10^5 were resuspended in 100 µl of binding buffer with 1 µl of annexin V-FITC solution (final concentration: 0.5 µg/ml) and 5 µl of PI. PI was used to gate for living cells and was added to each tube at a final concentration of 5 µg/ml. The cells were kept on ice for 10 min in the dark, and then added with 400 µl of binding buffer. The presence of viable (annexin V-negative and PI-negative), early apoptotic (annexin V-positive, PI-negative), and late apoptotic (annexin V-positive and PI-positive) cells were analyzed by flow cytometry. The extent of apoptosis in this experiment was quantified as percentage of annexin V-positive and PI-negative cells.

Superoxide and Peroxide Measurement
To determine the quercetin-induced changes in superoxide and peroxide contents in the hepatoma cells, the method of Carter et al. was used (18). Cells were washed twice with sterile Hank’s balanced salt solution (HBSS), then resuspended in sterile-filtered HBSS solution with Ca^2+ and Mg^2+ (GIBCO, Grand Island, NY) containing 0.22% glucose, 2 mM glutamine, and 1% bovine serum albumin. Cells were then added with either 20 µM 2’, 7’-dichlorofluorescin diacetate (DCFH-DA; Eastman Kodak Co., Rochester, NY) or 10 µM hydroethidine (HE; Molecular Probe, Eugene, OR) and incubated at 37°C for 30 min. At the end of incubation, cell suspensions were kept in an ice bath (4°C). PI (10 µg/ml) was added to the tubes for gating the viable cells containing DCF and the cells were kept on ice for 10 min. Samples were analyzed by flow cytometry. The trend of peroxide level after quercetin treatment was expressed as fold of increase (mean of fluorescence intensity of quercetin-treated group/mean of fluorescence intensity of control group).

High Performance Liquid Chromatography (HPLC) Analysis of Malondialdehyde (MDA)
To determine the quercetin-induced membrane lipid peroxidation in hepatoma cells, the method of Wong et al. was used (19). The cells were collected and frozen in –20°C, and the culture medium were collected and lyophilized. Lyophilized medium were dissolved in 1 ml 0.4 M phosphoric acid for 15 s. Frozen cells were homogenized in 0.5 ml of 0.4 M phosphoric acid for 15 s. Medium–phosphorid acid mixture in the amount of 100 µl or an aliquot (50 µl) of the cell homogenate was added with 750 µl of 0.4 M phosphoric acid and 250 µl of 42 mM thiobarbituric acid solution and boiled at 100°C for 60 min. The mixture was cooled in an ice bath for at least 10 min. Prior to HPLC analysis, an aliquot of the sample (500 µl) was neutralized with 500 µl of methanol-NaOH, centrifuged at 9,500 g for 5 min and 50 µl of the clear supernatant was injected. The HPLC system included a Kratos spectroflow 757 UV/VIS detector, a Rheodyne 7125 sample injector fitted with a 50 µl loop. The mobile phase was methanol/50 mM potassium phosphate buffer (pH 6.8; 4:6, vol/vol). The flow rate was 1.1 ml/min, 1, 1, 3, 3-tetraethoxypropane was used as the standard. The total
MDA level was the extracellular MDA (in medium) added with intracellular MDA and normalized with cell number. The total MDA level was expressed as $n$ mole/10$^6$ cells.

Statistical Analysis

All data were expressed as mean ± SE. The differences between groups were assessed using Student’s unpaired $t$-test. A $P < 0.05$ is considered as significant difference.

Results

Growth Inhibition of Quercetin in Hepatoma Cells

To determine the ability of quercetin to inhibit cell proliferation in two human hepatoma cell lines, cells were exposed to various concentrations of quercetin (0, 40, 60, 80 µM) for 24, 48, and 72 h. As shown in Fig. 1, significant growth inhibition was observed at 24, 48, 72 h in two hepatoma cell lines after treatment with 40, 60, 80 µM quercetin.

Cell Cycle and Annexin V Binding Analysis

Figure 2 shows that treatment with 40, 60, 80 µM quercetin for 72 h resulted in an increase in the apoptotic subG0-G1 phase cells from 3.5% to 10.8–32.7% and 1.9% to 18.9–26.1% in HA22T/VGH and HepG2 cells, respectively. The S phase of HA22T/VGH cells were increased (43.1–61.1%) as compared to control (19.1%), and a concomitant decrease of cells in the G0-G1 phase from 68.4% (control) to 24.1–38.3% at 72 h. Dose specifically decrease in the G0-G1 phase was observed on 40 µM quercetin-treated HepG2 cells. However, HepG2 cells did not present obvious changes at the G0-G1 or S phase as compared to control at 72 h after treatment with 60 or 80 µM quercetin. The early apoptotic marker annexin V binding was...
Figure 2. The effect of quercetin (Qu) on cell cycle progression at 72 h in HA22T/VGH and HepG2 cells. Cells were seeded at $5 \times 10^5$ cells per 25 cm$^2$ of tissue flask in the growth medium. The following day the cells were replenished with medium containing 40, 60, or 80 µM quercetin. After treatment for 72 h, cells were harvested and the cell cycle was analyzed by flow cytometry as described in Materials and Methods. The representative cell cycle progressions in quercetin-treated cells are from one of two independent experiments.
also significantly \((P < 0.05)\) increased in both cell lines at 72 h after treatment with 60 or 80 \(\mu M\) quercetin (Fig. 3).

**Effect of Quercetin on the Levels of Superoxide, Peroxide, and Lipid Peroxidation**

To examine whether the treatment of quercetin affected the levels of superoxide and peroxide in hepatoma cells, we examined the quercetin treated cells using HE and DCFH-DA as probes by flowcytometry analysis. Figure 4A shows that the superoxide and peroxide levels were increased in quercetin treated HA22T/VGH and HepG2 hepatoma cells. The superoxide level showed a 3.2-fold increased as compared to control at 72 h in 80 \(\mu M\) quercetin treated HA22T/VGH cells. However, the increased levels (1.1–1.7-fold) of superoxide in HepG2 cells were less than that of HA22T/VGH cells. The peroxide production was slightly increased in both quercetin-treated cells (Fig. 4B).

The 80 \(\mu M\) quercetin-induced lipid peroxidation was also significantly \((P < 0.05)\) higher than vehicle control in these two hepatoma cells (Fig. 5).
Enhancement of the Apoptotic Effect of Paclitaxel by Quercetin

It was demonstrated that ROS was involved in paclitaxel-induced apoptosis (20). To further investigate whether quercetin could enhance the apoptotic effect of paclitaxel, HA22T/VGH cells were pretreated with quercetin for 24 h and then replaced with fresh medium containing the paclitaxel for another 48 h. Annexin V binding of HA22T/VGH cells was analyzed by flow cytometry. Figure 6 shows that treatment with quercetin (40, 60, or 80 µM) for 24 h or paclitaxel (0.1 µM) for 48 h in HA22T/VGH cells, the levels of annexin V binding were 2.7%, 2.5%, 7.9%, and 6.9%, respectively. The levels of annexin V binding in 40, 60, or 80 µM quercetin combination with paclitaxel-treated cells were 10.9%, 22.7%, and 26.5%, respectively. These results clearly showed that quercetin enhanced the apoptotic effect of the paclitaxel.

Discussion

In this study, evidence supported that the elevation of ROS was involved in quercetin-induced cytotoxicity in human...
active metabolic production of O$_2^-$ would have to rely on tect cells from damage induced by ROS. Cancer cells with an...tions in hepatoma cells is not clear. The quercetin-induced dif-

increased the levels of malondialdehyde during apoptosis. The detailed mechanism of quercetin-induced growth inhibi-

different responses in hepatoma cell lines may relate to their...S phase was increased in quercetin-treated HA22T/VGH cells, but not HepG2 hepatoma cells. These differences are most likely due to the specific cell type or the concentration used of quercetin.

Quercetin has been reported to have both antioxidant and pro-oxidant (26,27) activities. Recent study shows that quercetin decreased manganese superoxide dismutase (Mn-SOD), glutathione peroxidase, and copper zinc superoxide dismutase (CuZn-SOD) mRNA expression levels in rat hepatoma H4IIE cells (28). Thus, it appeared that a decrease in SOD and glutathione peroxidase level was closely related with the ROS accumulation in hepatoma cells (28). SODs are essential enzymes that eliminate O$_2^-$ and thus protect cells from damage induced by ROS. Cancer cells with an active metabolic production of O$_2^-$ would have to rely on SOD for elimination of the superoxide radical. Many chemotherapeutic agents exert their toxic effects on cancer cells by producing free radical. The overproduction of ROS in cancer cells may exhaust the capacity of SOD and other antioxidant defenses. In this study, we have found that the level of ROS and malondialdehyde was increased in quercetin-treated hepatoma cells. These results correlated well with the quercetin-induced cytotoxic effects in human leukemic HL-60 cells (29), and similar results were also observed in isolated rat-liver nuclei (30). Quercetin has been found to inhibit glutathione reductase and glutathione S-transferase activities and lead to reduced cellular levels of glutathione (26,31). It has been repoted that quercetin may act as a cytotoxic pro-oxidant after its metabolic activation to semiquinone and quinoidal product (12). These studies strongly suggest that quercetin has pro-oxidant activity, and that was involved in the quercetin-induced apoptosis.

It also has been demonstrated that quercetin exert protective effects against hydrogen peroxide-induced cytotoxicity at the concentration as low as 10–25 µM in rat hepatoma cells. On the other hand, the quercetin induced cytotoxicity, DNA strand break, oligonucleosomal DNA fragmentation, and caspase activation at concentrations between 50 and 250 µM (32). This quercetin-induced cytotoxic pro-oxidation agrees well with our results. This might depend on its higher concentrations, and similar results were also found in Watjen et al. (32). The chemical stability of quercetin is usually unstable (33). In our study, the cytotoxic pro-oxidative activity after 72 h of exposure may have resulted from breakdown products of quercetin (such as superoxide) or to very downstream effects of other physiological processes and not directly to quercetin itself.

Quercetin is considered to be the most important flavonoid due to its ubiquity in the diet. Quercetin has selective effects on the cell growth and antioxidant defense system in normal versus transformed mouse hepatic cell lines (34) and can be considered as a chemoprevention and chemotherapeutic agent. Evaluating the effects of quercetin individually and in combination with chemotherapeutic agents may help identify the possible benefits of quercetin in cancer therapy. The combination might help to reduce the side-effects allowing the reduction of the administrated dose of chemotherapeutic agents. Paclitaxel has been found to increase the levels of superoxide, hydrogen peroxide, nitric oxide, oxidative DNA adducts, and cell with fragmented nuclei, and cellular total antioxidant capacity is a critical determinant of cellular sensitivity to paclitaxel (20). Our result showed that paclitaxel in combination with quercetin significantly enhanced the in vitro cytotoxic effect. This result suggests that the ROS generator, high dose quercetin, in combination with paclitaxel, is likely to produce additive or even synergetic effect and agrees well with the observations of paclitaxel in cancer cells (20). Although the causality of quercetin induced apoptosis of hepatoma cells needs further investigation, our in vitro results provide the basis for further study into the potential use of quercetin or quercetin in combination with chemotherapeutic agents for treatment of hepatoma.

![Figure 5](image-url)

**Figure 5.** The effect of quercetin on the lipid peroxidation in HA22T/VGH and HepG2 cells. The level of malondialdehyde (MDA) formation was assayed by HPLC in two hepatoma cell lines after treatment with 80 µM quercetin for 24–72 h. Data points represent mean ± S.E. of duplicate results from a representative experiment of two independent experiments. C, control; Qu, 80 µM quercetin; *, P < 0.05, indicates significant difference from control.
Figure 6. Enhancement of the apoptotic effect of paclitaxel by quercetin. A: The representative histogram of 80 µM quercetin in combination with 0.1 µM paclitaxel in HA22T/VGH cells. B: Cells were pretreated with quercetin (40, 60, or 80 µM) for 24 h, and then replated with or without paclitaxel (0.1 µM) for another 48 h. The apoptotic effect of each treatment was assayed by annexin V binding method. Data points are the mean ± SE of percentage of propidium iodide (PI) negative and Annexin V-FITC positive cells from two independent experiments. **, P < 0.05, represents significant difference from 60 µM quercetin or 0.1 µM paclitaxel only group; *, P < 0.05, represents significant difference from vehicle control.
Acknowledgments and Notes

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