Stabilization of p53 Is Involved in Quercetin-Induced Cell Cycle Arrest and Apoptosis in HepG2 Cells

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There is evidence for defects in the mechanisms that allow the activation of p53 in many of the cancers that retain wild-type p53. Reactivation of p53 has been suggested to be an effective strategy for cancer therapy in wild-type p53-retained tumor cells. In the present study, we attempted to reactivate p53 in HepG2 retaining wild-type p53 by quercetin, an ubiquitous bioactive plant flavonoid. Our results show that quercetin inhibited the proliferation of HepG2 cells through the induction of cell cycle arrest and apoptosis, as characterized by the cell cycle distribution and DNA fragmentation. Molecular data revealed that quercetin induced p53 phosphorylation and total p53 protein, but that it did not up-regulate p53 mRNA at the transcription level. Consequently, quercetin stimulated p21 expression and suppressed cyclin D1 expression in favor of cell cycle arrest. Quercetin also increased the ratio of Bax/Bcl-2 in favor of apoptosis. Quercetin inhibited p53 ubiquitination and extended the half-life (t1/2) of p53 from 74 to 184 min. Quercetin also inhibited p53 mRNA degradation at the post-transcription stage. Silencing p53 with p53 small interfering RNA (siRNA) significantly abrogated the p53-dependent gene expression and apoptotic induction. Taken together, our data demonstrate that quercetin stabilized p53 at both the mRNA and protein levels to reactivate p53-dependent cell cycle arrest and apoptosis in HepG2 cells.

Key words: quercetin; p53; cell cycle arrest; apoptosis; ubiquitination

The tumor suppressor protein, p53, plays a central role in tumorigenesis and cancer prevention. P53 can regulate cell cycle arrest, apoptosis and DNA repair in a variety of cells.1  The major downstream effectors of p53 include p21 and cyclin D1, which participate in cell cycle arrest,2,3 and Bax as well as Bcl-2, which trigger apoptosis.4,5 The diverse phosphorylation sites of p53 have been demonstrated to play important roles in the regulation of many cellular responses,6 of which the phosphorylation of p53 at serine 15 is an important target for p53 activation and stabilization.7 The importance of p53 in tumor suppression is highlighted by the observation that about half of all human cancers show evidence for the loss of normal p53 function due to mutation within the p53 gene. On the other hand, in many of the cancers that retain wild-type p53, there is evidence for defects in the mechanisms that allow the activation of p53. One of the principal reasons is the degradation of p53 which leads to the loss of p53 activity. Thus, stabilization of the p53 protein has been suggested to be an effective strategy for cancer therapy in wild-type p53 retained tumor cells.8,9 Recent reports have revealed that some small compounds could reactivate the p53 function to inhibit cancer cell proliferation through cell cycle arrest and/or apoptosis, which opens new possibilities to fight cancer.10 Quercetin, an ubiquitous bioactive plant flavonoid, has been shown to inhibit cell proliferation in several cancer cells. Investigations into the molecular mechanisms underlying the inhibition of cell proliferation by quercetin have shown that a treatment with quercetin triggered numerous cellular events such as p53 activation, cell cycle arrest at G1, and/or G2/M arrest and induction of caspase-mediated apoptosis in some cancer cells.11 However, the molecular mechanisms underlying the reactivation of p53 by quercetin to inhibit cell proliferation remain unclear.

In the present study, we used HepG2 cells harboring the wild-type p53 gene12 to investigate the ability of quercetin to inhibit cell proliferation, and further to clarify the mechanism. Our results indicate that quercetin induced p53-mediated cell cycle arrest and apoptosis.
in HepG2 through the stabilization of p53 mRNA and protein.

Materials and Methods

Materials and cell culture. Quercetin and cycloheximide (CHX) were purchased from Sigma, while MG132 and actinomycin D were from Calbiochem. The antibodies against p53 (DO-1), p21 (H-164), cyclin D1 (DCS6), α-tubulin (B-7), Bax (B-9), Bcl-2 (C-2) and ubiquitin (P4D1) were from Santa Cruz Biotechnology. The antibodies against phospho-p53 at ser 15 (#9284), ubiquitin (P4D1), and anti-mouse secondary antibodies (#7076) were from Cell Signaling Technology. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS.

Cell survival assay. The cell survival rate was measured by an MTT assay as described previously. Cell viability is expressed as the optical density ratio of the treatment to control. The IC50 value is defined as the concentration required for 50% reduction in cell survival.

DNA fragmentation detection. DNA was extracted and detected as described previously. A flow cytometric analysis of DNA fragmentation and cell cycle distribution was conducted by fixing HepG2 in 70% ethanol at −20°C overnight, and then washing and darkly staining the cells with 25 μg/ml of propidium iodide for 30min. Fluorescence was evaluated according to a linear scale (Partec), and the cell cycle distribution was analyzed with Flo Max Software (Partec). Cells with a DNA content was designated as being in the G1, S or G2/M phase of the cell cycle. The number of cells in each compartment of the cell cycle is expressed as a percentage of the total number of cells used.

RNA extraction and RT-PCR. Total RNA was extracted with an Isogen RNA kit (Nippon Gene) according to the manufacture’s manual. The PCR primers used for the human p53 gene were 5'-CCCTCCAGAAAACCTACCA-3' (forward) and 5'-TCATAGGGCACCACCACT-3' (reverse), which are located in exons 4 and 6, and amplify a 371-bp fragment. The PCR primers for the human GAPDH gene were 5'-GACCCCTTACCTGACCT-CAAC-3' (forward) and 5'-CATACCAAGGAAAT-GAGCTTG-3' (reverse) which amplify a 843-bp fragment. RT-PCR was performed with Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech). Total RNA (250 ng) was used to reverse mRNA into cDNA at 42°C for 30 min with oligo (dT)12-18 primers. Amplification was done at 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec with 26 cycles for p53 and 24 cycles for GAPDH. The PCR products were separated on 2% agarose gel and quantified with Imager software (Taitec).

Immunoprecipitation and Western blotting analysis. Whole cell lysates containing 1 mg of proteins were pre-cleared with protein A-sepharose beads (Amersham Pharmacia Biotech) for 1 h, and then incubated with 2 μg of the anti-p53 antibody for 4 h. The immunoprecipitated complexes were washed, and finally boiled in an SDS sample buffer for 5 min. Either the immunoprecipitated products or whole cell lysates (40 μg of proteins for detecting p-p53, p53, p21, α-tubulin and cyclinD1, and 80 μg of proteins for detecting p-Bcl2, Bcl2, Bax and caspase3) were run on 8% or 12% SDS–PAGE and then transferred to a PVDF membrane (Amersham Pharmacia Biotech). The membrane was incubated overnight with the specific antibody at 4°C, and further incubated for 1 h with the HRP-conjugated secondary antibody. Bound antibodies were detected by the ECL system. For cycloheximide (CHX) chase experiment, HepG2 cells were pre-treated with or without 80 μM quercetin for 4 h, and the medium was then replaced with a medium containing CHX (5 μg/ml) for 0–120 min. Total p53 protein was detected by Western blotting with the p53 antibody. The half-life (t1/2) of p53 protein was calculated according to the one-phase exponential decay formula.

Transfection of siRNA. Pre-designed siRNA against human p53 (catalog no. 115762) was purchased from QIAGEN, and control scrambled siRNA (catalog no. 4611) was purchased from Ambion. HepG2 cells (3 × 105) were transfected with 100 nM p53 siRNA or the scrambled duplex by using LipofectAMINE2000 (Invitrogen). After 24 h of incubation, a fresh medium was added and culture continued for another 48 h. The cells were then treated with 80 μM quercetin for an additional 12 or 48 h.

Statistical analyses. The difference between the treated and control cells were analyzed by Student’s t-test, a probability of P < 0.05 being considered significant.

Results

Quercetin inhibits the proliferation of HepG2 cells through the induction of cell cycle arrest and apoptosis

To test the effect of quercetin on the proliferation of HepG2 cells, the cells were treated with different concentrations of quercetin for 0–48 h, and the surviving cells were then investigated by an MTT assay. As shown in Fig. 1A, quercetin inhibited cell proliferation in a dose- and time-dependent manner with an IC50 (50% inhibitory concentration) value of 76.1 μM after 48 h of treatment. To evaluate whether this quercetin-induced inhibition of cell proliferation was due to cell cycle arrest and/or apoptotic death, we first examined the cell
distribution in different cell cycles. As shown in Fig. 1B, cells in the S phase were significantly decreased, and cells in the G2/M phase were significantly increased by the quercetin treatment. Thus, quercetin caused cell cycle arrest in the HepG2 cells. Next, we characterized apoptotic death in the quercetin-treated cells. As shown in Fig. 1C, clear DNA fragmentation was detected during the 36–48 h period. Taken together, we conclude that quercetin inhibited the proliferation of HepG2 cells through cell cycle arrest and apoptotic death induction.

**Quercetin activates p53 and p53-dependent genes**

The tumor suppressor, p53, plays a critical role in cell cycle arrest and apoptosis. HepG2 cells have been reported to harbor the wild-type p53 gene. To examine whether quercetin induced the activation of p53, HepG2 cells were treated with 80 µM quercetin for 12–48 h. As shown in Fig. 2, phosphorylation and the total amount of p53 protein were increased during the 12–36 h period in the quercetin-treated cells. We next investigated the expression of p53 downstream effectors such as p21 and cyclin D1, and Bax and Bcl-2, which are key factors involved in cell cycle arrest and apoptotic death. Up-regulation of p21 expression and down-regulation of cyclin D1 expression were observed in the 80 µM quercetin-treated cells during the 12–48 h period. On the other hand, no significant change in p53-dependent gene expression such as p21 and cyclin D1 was apparent in the control cells during the 0–48 h period (data not shown). These results suggest that quercetin may have induced p53-mediated cell cycle arrest.

Moreover, a decrease in phosphorylated and total protein of Bcl-2, and increase in Bax protein were also observed with such a treatment. Since an alteration in the ratio of Bax/Bcl-2 is known to initiate caspase
To clarify the effect of quercetin on p53 regulated by an ubiquitin-dependent protein degradation and mRNA levels, we examined the morphology of HepG2 cells transfected with p53 siRNA molecules into HepG2 cells to block the p53 protein expression and p53-dependent events. As shown in Fig. 4A, p53 siRNA reduced the basal as well as quercetin-induced p53 protein and p53 phosphorylation levels (lanes 5 and 6). Moreover, up-regulation of p21 and activation of caspase 3 induced by quercetin were also abrogated after transfection with p53 siRNA. As a control, scrambled siRNAs had no effect on these factors (lanes 3 and 4). To confirm these results, we further examined the morphology of HepG2 cells transfected with p53 siRNA. As shown in Fig. 4B, transfection with p53 siRNA abrogated the inhibition of cell proliferation induced by quercetin. Scrambled siRNAs had no such effect, compared to the control cells. A flow cytometric assay revealed that, in quercetin-treated cells, transfection with p53 siRNA decreased the proportion of hypodiploid phase (Sub G1) cells by 1.3-fold. As a

In order to clarify whether quercetin-induced p53 protein also depended upon transcriptional regulation, we performed an RT-PCR analysis of p53 mRNA. A treatment with 80μM quercetin did not affect the p53 mRNA level after 12 h (Fig. 3C). To clarify the p53 mRNA stability, we pretreated HepG2 cells with 5μg/ml of actinomycin D, a transcriptional inhibitor, for 1 h and then cultured for another 12 h. Treatment with actinomycin D decreased the p53 mRNA level (Fig. 3C, lane 3). However, co-treatment with actinomycin D and quercetin in the same experiment kept p53 mRNA stable (Fig. 3C, lane 4). As a control, GAPDH showed no change with such treatment. These results indicate that quercetin did not up-regulate p53 at the transcriptional level, but stabilized p53 mRNA at the post-transcriptional level.

Activation of p53 is essential for quercetin-induced apoptosis

To confirm whether the activation of p53 was essential for quercetin-induced apoptosis, we transfected p53 siRNA molecules into HepG2 cells to block the p53 protein expression and p53-dependent events. As shown in Fig. 4A, p53 siRNA reduced the basal as well as quercetin-induced p53 protein and p53 phosphorylation levels (lanes 5 and 6). Moreover, up-regulation of p21 and activation of caspase 3 induced by quercetin were also abrogated after transfection with p53 siRNA. As a control, scrambled siRNAs had no effect on these factors (lanes 3 and 4). To confirm these results, we further examined the morphology of HepG2 cells transfected with p53 siRNA. As shown in Fig. 4B, transfection with p53 siRNA abrogated the inhibition of cell proliferation induced by quercetin. Scrambled siRNAs had no such effect, compared to the control cells. A flow cytometric assay revealed that, in quercetin-treated cells, transfection with p53 siRNA decreased the proportion of hypodiploid phase (Sub G1) cells by 1.3-fold. As a

Quercetin stabilizes p53 protein at both the protein and mRNA levels

Accumulated data have revealed that p53 protein is regulated by an ubiquitin-dependent protein degradation system. To clarify the effect of quercetin on p53 stability, HepG2 cells were pretreated or not with 80μM quercetin for 4h; no difference in the amount of p53 protein was apparent between the treated and control cells (data not shown). We then treated HepG2 cells with 5μg/ml of CHX, a protein synthesis inhibitor, for 15–120 min. After harvesting, p53 protein was detected with its antibody and the density was quantified. The half-reduction time (t1/2) from protein decay experiments was then calculated. As shown in Fig. 3A, the treatment with 80μM quercetin extended the half-life (t1/2) of p53 protein from 74 min to 184 min, 2.4 times longer. This result suggests that quercetin may have stabilized the p53 protein at the post-translational level. We next examined p53 ubiquitination after a treatment with 26S proteasome specific inhibitor MG132 in the presence or absence of quercetin. As shown in Fig. 3B, total p53 protein and p53 phosphorylation at serine 15 were increased in the cells treated with quercetin (input, lane 2) and in those co-treated with quercetin and MG132 (input, lane 4). After immunoprecipitation with the p53 antibody, ubiquitination of p53 was markedly reduced in the cells co-treated with quercetin and MG132 (Fig. 3B, IP, lane 4). These results revealed that stabilization of p53 in the quercetin-treated cells was due to the inhibitory effect of quercetin on p53 ubiquitination.

In order to clarify whether quercetin-induced p53 protein also depended upon transcriptional regulation, we performed an RT-PCR analysis of p53 mRNA. A treatment with 80μM quercetin did not affect the p53 mRNA level after 12 h (Fig. 3C). To clarify the p53 mRNA stability, we pretreated HepG2 cells with 5μg/ml of actinomycin D, a transcriptional inhibitor, for 1 h and then cultured for another 12 h. Treatment with actinomycin D decreased the p53 mRNA level (Fig. 3C, lane 3). However, co-treatment with actinomycin D and quercetin in the same experiment kept p53 mRNA stable (Fig. 3C, lane 4). As a control, GAPDH showed no change with such treatment. These results indicate that quercetin did not up-regulate p53 at the transcriptional level, but stabilized p53 mRNA at the post-transcriptional level.

Activation of p53 is essential for quercetin-induced apoptosis

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![Fig. 2. Quercetin Induces the Expression of p53 and p53-Dependent Genes.](image-url)
control, Sub G1 cells were 3.1-fold higher in the cells transfected with scrambled siRNA, and 2.8-fold higher in the cells without transfection (Fig. 4C). We thus conclude that p53 was required for the inhibition of cell proliferation induced by quercetin in HepG2 cells.

**Discussion**

To identify the effect of quercetin on cancers that retain wild-type p53, we used HepG2 cells, a cell line of human hepatoma harboring wild-type p53. Treatment
Silencing \( p53 \) with quercetin resulted in \( p53 \)-mediated cell cycle arrest. In response to the quercetin treatment, cell cycle was arrested in G2/M with a parallel rise in \( p21 \) level, and reciprocal fall in cyclin D1 (Fig. 2), suggesting that the quercetin-induced inhibition of cell proliferation involved cell cycle arrest.

Bax is apoptotic protein and Bcl-2 is anti-apoptotic protein. A change in the ratio of Bax/Bcl-2 is known to initiate caspase signaling. Treatment with quercetin increased the Bax level, and reduced Bcl-2 phosphorylation and total protein (Fig. 2). In other words, treatment with quercetin increased the ratio of Bax/Bcl-2 in favor of apoptosis, and caused consequent activation of caspase-3. These results suggest that quercetin might have induced \( p53 \)-mediated apoptotic death.

It is worth noting that the treatment with quercetin extended the half-life (1/\( t_{1/2} \)) of \( p53 \) protein from 74 min to 184 min, 2.4 times longer (Fig. 3A). This result that quercetin stabilized \( p53 \) protein at the post-translational level suggests a possibility that quercetin may inhibit the ubiquitination of \( p53 \). To prove this hypothesis, we examined the ubiquitination of \( p53 \) by immunoprecipitation after treating with 26S proteasome specific inhibitor MG132 in the presence or absence of quercetin. A marked reduction in \( p53 \) ubiquitination was apparent from the co-treatment with quercetin and MG132 (Fig. 3B, IP, lane 4). Therefore, quercetin might stabilize \( p53 \) protein by inhibiting \( p53 \) ubiquitination and protein turnover at the post-translation level. The \( p53 \) protein stabilized by quercetin may ensure \( p53 \)-dependent actions such as cell cycle arrest and apoptosis.

Furthermore, an RT-PCR analysis confirmed that quercetin did not up-regulate \( p53 \) mRNA at the transcriptional level, but could stabilize \( p53 \) mRNA at the post-transcriptional level (Fig. 3C). Several studies have suggested that \( p53 \) mRNA can be stabilized by associating with RNA-binding protein HuR which is predominantly nuclear in unstimulated cells but translocates to the cytoplasm in response to various stimuli. Cellular polyamine has been found to modulate the HuR function. Ornithine decarboxylase (ODC) is a rate-limiting enzyme for polyamine synthesis, and its specific inhibitor such as \( \alpha \)-difluoromethylornithine dramatically stabilizes \( p53 \) mRNA and increases the rate of newly synthesized \( p53 \) protein by increasing the cytoplasmic level of HuR with the depletion of cellular polyamine. Quercetin has been reported to inhibit the ODC activity in mouse skin. Recent results with different cell lines indicate that quercetin was a stronger inhibitor of ODC activity with IC\(_{50}\) value of 1.3 \( \mu \)M. Based on our results and other information, we consider that quercetin may stabilize \( p53 \) mRNA by inhibiting ODC activity. In other words, quercetin may inhibit ODC activity and reduce cellular polyamine. This
depleted cellular polyamine can increase the cytoplasmic level of HuR to stabilize p53 mRNA. It is necessary to confirm these mechanisms in future work.

Although the molecular target of quercetin to stabilize p53 protein has not yet been identified in the present studies, recent reports have shown that the stability of p53 protein was tightly regulated by such ubiquitin ligases as HDM2, Cop1, Pirh2, and ARF-BP1. For example, the inactive form of p53 has been found to bind to HDM2 protein, which is an E3 ubiquitin ligase for p53, and to promote the ubiquitination and degradation of p53 through the 26S proteasome protein degradation system. Thus, p53 dissociation from HDM2 is considered to be the critical step for the stabilization of p53 and p53-dependent gene expression. In respect of p53 stabilization, phosphorylation of p53 at serine 15 leads to the dissociation of HDM2 from p53, and then to the induction of transcriptional activities of p53. As an example, genistein, a natural isoflavone present in soybeans, has been found to down-regulate HDM2 at least partially by enhancing the degradation of HDM2. Based on our data and information on p53 reactivation mechanisms, we consider that quercetin may target the molecule(s) of ubiquitin ligase to stabilize p53 protein through a post-translational mechanism, this deserving further investigation.

It has been reported that quercetin could induce ROS including H2O2 in human leukemia cells such as HL-60 and MOLT-4. Generated ROS can induce apoptosis by a mitochondrial dysfunction pathway such as loss of the mitochondrial membrane potential and cytochrome c release from the mitochondrion into cytoplasm. Moreover, oxidative stress may influence p53 through the activation of PKCδ and then cause apoptosis. For these reasons, we measured the H2O2 level in quercetin-treated HepG2 cells by a dichlorofluorescin (DCFH) assay and found that there was no significant difference from the control cells (data not shown). Our results are in agreement with a recent study in which treatment with quercetin (100 μM) did not generate ROS in HepG2 cells.

Recent studies have clarified that myeloperoxidase present in HL-60 cells catalyzed the catechol structure of polyphenols to the oxidized form; thus, polyphenols are considered to function as pro-oxidants in the leucocytes. Based on our data and those in other reports, we consider that the reactivation of p53 by quercetin might be independent of ROS production in HepG2 cells.

In summary, our results indicate that quercetin, an ubiquitous bioactive plant flavonoid, induced p53-mediated cell cycle arrest and apoptosis in HepG2 through the stabilization of p53 mRNA and protein. These findings will provide new insight into the cancer chemoprevention properties of plant flavonoids.

Acknowledgments

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References


