INHIBITION OF LUNG CANCER CELL GROWTH BY QUERCETIN GLUCURONIDES VIA G2/M ARREST AND INDUCTION OF APOPTOSIS

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ABSTRACT:
Lung cancer is the leading cause of cancer death in many developed countries, including Taiwan. Quercetin, a widely distributed bioflavonoid, is well known to induce growth inhibition in a variety of human cancer cells. Quercetin glucuronides are the main circulating metabolites after dietary supplements with quercetin in humans. However, there is little information available as to how quercetin glucuronides affect human cancer cells. We investigated the effects of quercetin glucuronides in a human lung cancer cell line NCI-H209. We checked the cell viability, cell cycle checkpoint proteins, pro- and antiapoptotic proteins, caspase-3 activity, and gene expression by flow cytometry and Western blot. The viability of cells decreased in a dose- and time-dependent manner. Cell cycle analysis revealed a significant increase of the proportion of cells in G2/M phase and subG0/G1 phase (corresponding to apoptotic cells). Moreover, quercetin glucuronides increased the expressions of cyclin B, Cdc25c-ser-216-p, and Wee1 proteins, indicating the G2/M arrest. We also demonstrated a concurrent decrease of the mitochondrial membrane potential, release of cytochrome c, up-regulation of Bax, down-regulation of Bcl-2, and activation of caspase-3, and subsequently, cleavage of poly(ADP-ribose) polymerase. In addition, quercetin glucuronide-induced apoptosis was totally blocked by the broad-spectrum caspase inhibitor benzoxycarbonyl-Val-Ala-Asp fluoromethylketone. Taken together, we demonstrated that quercetin glucuronides inhibited proliferation through G2/M arrest of the cell cycle and induced apoptosis via caspase-3 cascade in the human lung cancer cell line NCI-H209. Delineation of the biological effects of specific major quercetin metabolites on chemotherapeutic potential or chemoprevention of human cancers warrants further investigation.

Lung cancer is the leading cause of cancer death in many developed countries, including Taiwan, in which 20% of cancer deaths have been caused by lung cancer (Greenlee et al., 2000). Small-cell lung carcinoma (SCLC), a form of lung cancer characterized by a neuroendocrine phenotype, represents approximately 20% of primary lung cancers and exhibits the most malignant phenotype of lung cancer (Chua et al., 2004). Most SCLC patients are not suitable for surgery, and the standard treatment for SCLC is chemotherapy or radiotherapy (Chua et al., 2004); however, the treatment outcome of SCLC or other types of lung cancer is far from satisfactory.

Flavonoids are naturally occurring polyphenolic compounds and have profound pharmacological properties, and a daily intake of flavonoids is associated with a lower risk of cancer (Middleton et al., 2000). Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the major dietary flavonoids particularly abundant in fruits and vegetables (Hertog et al., 1993). Analysis of plasma of volunteers fed with a quercetin-supplemented diet shows that quercetin is mainly circulating as quercetin glucuronides (Fig. 1) (Manach et al., 2004). Most of the biological studies for assessing the properties of quercetin are performed on quercetin (aglycone), and there is limited information available regarding the biological effects of quercetin glucuronides.

Quercetin has been shown to have diverse biological activities, including antiproliferative and apoptotic effects (Choi et al., 2001), although the mechanisms are still obscure. Quercetin treatment caused cell cycle arrest either at the G1/S or G2/M transition, depending on cell type (Choi et al., 2001; Nguyen et al., 2004; Ong et al., 2004). Moreover, quercetin-mediated apoptosis may be related to many factors such as stress proteins, disruption of microtubules, nuclear factor-kB, Cox-2, p53, survivin, c-Jun NH2-terminal kinase, mitogen-activated protein kinase kinase-extracellular signal-regulated kinase, Bcl-2 family proteins, heat shock proteins, DNA topoisomerase II, release of cytochrome c, and activation of caspases (Yoshida et al., 1990; Orzechowski et al., 2000; Choi et al., 2001; Jakubowicz-Gil et al., 2002; Kaneuchi et al., 2003; Cheong et al., 2004; Kuo et al., 2004; Nguyen et al., 2004; Ong et al., 2004). Quercetin has been reported to...

ABBREVIATIONS: SCLC, small-cell lung carcinoma; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate-buffered saline; z-VAD-fmk, benzoxycarbonyl-Val-Ala-Asp fluoromethylketone; PARP, poly(ADP-ribose) polymerase; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; CDK, cyclin-dependent kinase; IAP, inhibitor of apoptosis protein; ER, endoplasmic reticulum.
inhibit the growth of various human cancers, including leukemia, breast, esophagus, colon, prostate, nasopharyngeal, endometrial, and lung cancers (Yoshida et al., 1990; Orzechowski et al., 2000; Choi et al., 2001; Jakubowicz-Gil et al., 2002; Kanecuchi et al., 2003; Cheong et al., 2004; Kuo et al., 2004; Nguyen et al., 2004; Ong et al., 2004). It is imperative to investigate the biological effects of quercetin glucuronides that are more close to the in vivo situation. Although quercetin-induced apoptosis has been documented in human lung cancer cells (Kuo et al., 2004; Nguyen et al., 2004), there are no reports addressing the molecular mechanisms of quercetin glucuronide-induced apoptosis in human lung cancer cells. Here, we report the findings that quercetin glucuronides inhibited cell proliferation through cell cycle arrest and induction of apoptosis in human lung cancer NCI-H209 cells.

Materials and Methods

Materials. Quercetin, catalase, ribonuclease-A, trypan blue, Tris-HCl, Triton X-100, and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO). Potassium phosphate, dimethyl sulfoxide (DMSO), and Trizma base were purchased from Merck Co. (Darmstadt, Germany). F12K medium, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and glutamine were obtained from Invitrogen (Carlsbad, CA). Caspase-3 activity assay kit was purchased from Roche Diagnostics (Mannheim, Germany).

Preparation of Quercetin Glucuronides. Quercetin glucuronides were prepared from the serum of rabbits that were administrated with quercetin, and the concentration of quercetin glucuronides was determined by high-performance liquid chromatography after hydrolysis with β-glucuronidase as described previously (Wittig et al., 2001; Kuo et al., 2002).

Human Lung Cancer Cell Line NCI-H209. NCI-H209 is a SCLC cell line procured from The Wellcome Trust Sanger Institute (Cambridge, UK), and we purchased it from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately transferred into 75-cm² tissue culture flasks and grown at 37°C under a humidified 5% CO₂ and 95% air at one atmosphere in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10 ng/ml penicillin and 10 ng/ml streptomycin), and 1% glutamine.

Cell Viability Determination by Using Trypan Blue Exclusion and Flow Cytometry. NCI-H209 cells were plated in 12-well plates at a density of 5 × 10⁵ cells/well and grown for 24 h. They were then added with different concentrations of quercetin glucuronides to reach final concentrations of 0, 0.5, 1, 2.5, 5, and 10 μM, while only adding DMSO (solvent) for the control and grown at 37°C, 5% CO₂ and 95% air for a different period of time. For determination of cell viability, the trypan blue exclusion protocol was used. Briefly, approximately 10 μl of cell suspensions in PBS were mixed with 40 μl of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer (Pettit et al., 1996) or using flow cytometric assay as described previously (Lee et al., 2003).

Determination of DNA Fragmentation of NCI-H209 Cells by Gel Electrophoresis. NCI-H209 cells were plated in six-well plates at a density of 5 × 10⁵ cells/well and grown for 24 h. They were then added with 50 and 100 μM quercetin glucuronides as described above, while only adding DMSO (solvent) for the control regimen and grown at 37°C in a humidified 5% CO₂ and 95% air for 48 h. DNA fragmentation was assessed by electrophoresis assay as described previously. The DNA was prepared using G-NOME DNA isolation kit protocol (BIO 101, La Jolla, CA) as described in previous studies (Chung, 1999).

Analysis of DNA Content by Flow Cytometry in NCI-H209 Cells. Approximately 5 × 10⁵ cells/well of NCI-H209 cells in six-well plate were incubated with concentrations (0, 0.5, 1, 2.5, 5, and 10 μM) of quercetin glucuronides for different time periods, and then the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) by putting 70% ethanol (in PBS) in ice overnight and were then resuspended in PBS containing 40 μg/ml propidium iodide and 0.1 mg/ml RNase (Sigma-Aldrich) and 0.1% Triton X-100 in a dark room. After 30 min at 37°C, the cells were analyzed on a flow cytometer (BD Biosciences, San Jose, CA) equipped with an argon laser at 488 nm wavelength. The cell cycle was then analyzed (Ormerod, 1990).
Detection of Reactive Oxygen Species (ROS) in NCI-H209 Cells by Flow Cytometry. The level of ROS of the NCI-H209 cells was determined by flow cytometry (Becton Dickinson FACS Calibur; BD Biosciences), using 2,7-dichlorodihydrofluorescein diacetate (Sigma-Aldrich). NCI-H209 cells were treated with or without various concentrations (0.5, 1, 2.5, 5, and 10 μM) of quercetin glucuronides for 24 h, and the levels of ROS were determined. The cells were harvested and washed twice, resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) and incubated at 37°C for 30 min and analyzed by flow cytometry (Kalbacova et al., 2003).

Detection of Mitochondrial Membrane Potential by Flow Cytometry. The mitochondrial membrane potential of the NCI-H209 cells was determined by flow cytometry (Becton Dickinson FACS Calibur; BD Biosciences), using the DiOC₆ (4 mol/l). NCI-H209 cells were treated with or without various concentrations (0.5, 1, 2.5, 5, and 10 μM) of quercetin glucuronides for 24 h, and the levels of ROS were determined. The cells were harvested and washed twice, resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (10 μM) and incubated at 37°C for 30 min and analyzed by flow cytometry (Kalbacova et al., 2003).

Determination of Caspase-3 Activity in NCI-H209 Cells. NCI-H209 cells were plated in 12-well plates at a density of 5 × 10⁵ cells/well and grown for 24 h. Various concentrations of quercetin glucuronides were added to the medium, and the cells were grown at 37°C in a humidified 5% CO₂ for 12 h. Approximately 5 × 10⁶ cells were lysed in the lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 mg/ml leupeptin) for 30 min at 4°C followed by centrifugation at 10,000g for 30 min. For caspase-3 activity determination, 50-μl reaction mixtures with fluorogenic peptide substrates were specifically used for caspase-3. The peptide substrate
(200 μM) was incubated at 37°C with cytosolic extracts (15 μg of total protein) in the reaction buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, and 0.1% 3-[3-chloroamidopropyl] dimethylammonio-1-propanesulfonate). Fluorescence was determined after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent; Thermo Electron Corporation, Waltham, MA) (Lu et al., 2004).

Effect of Caspase Inhibitor z-VAD-fmk on Caspase-3 Activity and Apoptosis. To examine whether or not caspase-3 activation is involved in the apoptosis triggered by quercetin glucuronides, NCI-H209 cells (5 × 10⁵ cells/ml) were treated with 0, 0.5, 1, 2.5, 5, and 10 μM quercetin glucuronides for 24 h, and then cytosolic fraction and total protein were prepared and determined as described under Materials and Methods. Then, we measured the levels of Wee1, Cdc25C-ser-216-p, CDK1, and cyclin B expressions by Western blotting.

Poly(ADP-Ribose) Monoclonal Antibody Assay for Apoptosis of NCI-H209 Cells. NCI-H209 cells were plated in 12-well plates at a density of 2 × 10⁵ cells/well and grown for 24 h. Various concentrations of quercetin glucuronides were added to the medium, and the cells were grown at 37°C in a humidified 5% CO₂ for 24 h for poly(ADP-ribose) monoclonal antibody assay (Alexis, San Diego, CA) as described in previous studies (Ormerod, 1990; Ferreira et al., 2002).
Western Blotting for Examination of Effect of Quercetin Glucuronides on the Expression of Apoptosis-Related Proteins. Total proteins were collected from human lung cancer NCI-H209 cells treated with quercetin glucuronides for 48 h. Western blotting was used to examine the expression levels of the apoptosis-related proteins including p21<sup>WAF1/CIP1</sup>, Cdc25C-ser-216-p (phosphorylation of Cdc25C on serine-216), Bax, Bad, Bcl-2, Bcl-x<sub>L</sub>, cytochrome <em>c</em>, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP) by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Ferreira et al., 2002).

**Statistical Analysis.** Student’s <em>t</em> test was used to analyze the statistical significance of the differences between the quercetin glucuronide-treated and control groups.

**Results**

Effects of Quercetin Glucuronides on Cell Viability of Lung Cancer NCI-H209 Cells. For control group, the data indicated that <2% of NCI-H209 cells were stained by trypan blue or propidium iodide when they were incubated in medium containing 10% FBS only. In the presence of quercetin glucuronides (0.5, 1, 2.5, 5, and 10 <em>M</em>), the dye density of cultured cells was increased by the increase of the time and concentration used, suggesting that quercetin glucuronides exerted a dose-dependent cytotoxic effect on NCI-H209 cells (Fig. 2, A and B). The IC<sub>50</sub> value of quercetin glucuronides was approximately 34.8 <em>M</em>.

Quercetin Glucuronides Induced Cell Cycle Arrest and Apoptosis in NCI-H209 Cells. The results indicated that during the 48-h time period, quercetin glucuronides increased the percentage of S and <em>G</em><sub>2</sub>/M phases, and the percentage of <em>G</em><sub>0</sub>/G<sub>1</sub> phase was decreased. Non-treated control cells showed a typical pattern of DNA content that reflected <em>G</em><sub>0</sub>/G<sub>1</sub>, S, and <em>G</em><sub>2</sub>/M phases of the cell cycle. The quercetin glucuronide-treated cells showed a typical pattern of DNA content that reflected <em>G</em><sub>0</sub>/G<sub>1</sub>, S, and <em>G</em><sub>2</sub>/M phases of the cell cycle together with a sub<em>G</em><sub>0</sub>/G<sub>1</sub> phase (corresponding to apoptotic cells) as shown in Fig. 3, A and B. Quercetin glucuronides induced a distinct sub<em>G</em><sub>0</sub>/G<sub>1</sub> peak, which represents the population of apoptotic cells. The percentage of apoptosis in various concentrations of quercetin glucuronides are shown in Fig. 3C.

**Flow cytometric analysis of mitochondrial membrane potential in human lung cancer NCI-H209 cells with or without treatment with quercetin glucuronides for 24 h**

**TABLE 1**

<table>
<thead>
<tr>
<th>Quercetin Glucuronides</th>
<th>Percentage of Cells Stained by DiO&lt;sub&gt;L6&lt;/sub&gt;</th>
<th>S.D.</th>
<th>&lt;sup&gt;*&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>0 (control)</td>
<td>96.1 ± 9.2</td>
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<td></td>
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<tr>
<td>0.5 &lt;em&gt;M&lt;/em&gt;</td>
<td>88.2 ± 8.1</td>
<td></td>
<td></td>
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<tr>
<td>1 &lt;em&gt;M&lt;/em&gt;</td>
<td>68.7 ± 7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 &lt;em&gt;M&lt;/em&gt;</td>
<td>44.6 ± 5.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 &lt;em&gt;M&lt;/em&gt;</td>
<td>29.6 ± 3.8&lt;sup&gt;*&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
<td>10 &lt;em&gt;M&lt;/em&gt;</td>
<td>12.8 ± 1.9&lt;sup&gt;*&lt;/sup&gt;</td>
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<sup>*</sup> Difference is significant between quercetin glucuronide-treated and control, <em>P</em> < 0.05.

Inhibition of Quercetin Glucuronide-Induced Caspase Activation and Apoptosis by z-VAD-fmk. These experiments were performed to examine whether caspase-3 activation is involved in the apoptosis triggered by quercetin glucuronides. The results indicate that quercetin glucuronides increased caspase-3 activity, and these effects are dose- and time-dependent (Fig. 6, A and B). NCI-H209 cells had been pretreated with the cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to the treatment with quercetin glucuronides. The caspase inhibitor decreased caspase-3 activity (Fig. 6C). After treatment with quercetin glucuronides and z-VAD-fmk in NCI-H209 cells, inhibition of quercetin glucuronide-mediated caspase-3 activation was accompanied by the marked attenuation of quercetin glucuronide-induced apoptotic cell death (Fig. 6, C and D). The results revealed that activation of caspase-3 contributes to quercetin glucuronide-induced apoptosis in NCI-H209 cells.

### Statistical Analysis

The change in ROS levels of NCI-H209 cells in response to varying concentrations of quercetin glucuronides was studied by staining cells with DiO<sub>L6</sub>. The results revealed that mitochondrial membrane potential was decreased in NCI-H209 cells after treatment with quercetin glucuronides. This effect was dose-dependent.
Poly(ADP-Ribose) Monoclonal Antibody Assay for Apoptosis in NCI-H209 Cells. Apoptosis of NCI-H209 cells were also determined by poly(ADP-ribose) fluorescence staining. When the apoptosis occurs to the cells’ DNA, cells might produce PARP for repair of damaged DNA, and the enzyme could be recognized by the antibody. Figure 7 shows PARP positive fluorescence at 0.5, 1, 2.5, 5, and 10 μM quercetin glucuronides. The results also showed a higher number of cells being stained in response to higher concentrations of quercetin glucuronides.

Effect of Quercetin Glucuronides on the Production of p21\textsuperscript{CIP1/WAF1}, Bak, Bax, Cytochrome c, Bcl-2, and Caspase-3 from NCI-H209 Cells. Total proteins were prepared and followed by Western blotting, and the results from SDS-PAGE are presented in Fig. 8. The results also showed that quercetin glucuronides increased p21\textsuperscript{CIP1/WAF1}, Bak, Bax, cytochrome c, and caspase-3 levels but decreased Bcl-2 levels. These findings suggest that quercetin glucuronides induced apoptosis through these molecular events.

Discussion

In the past years, the strategy for killing cancer cells through the induction of apoptosis has been extensively studied (Ferreira et al., 2002; Norbury and Zhivotovsky, 2004). In general, the initiation of cellular apoptosis is through two distinct pathways: one is the...
extrinsic pathway involved the death receptor signaling, and the other is the intrinsic pathway involved in the mitochondrial cascades (Green and Amarante-Mendes, 1998; Kroemer and Reed, 2000; Henry-Mowatt et al., 2004). Activation of either one of the pathways by cleavage of the procaspase-8 or -9 will result in further downstream activation of caspase-3, caspase-activated DNase, and finally lead to DNA fragmentation (Ferreira et al., 2002; Norbury and Zhivotovsky, 2004). Activation of caspase-3 is often considered as the point-of-no-return in the apoptotic signaling cascade (Green and Amarante-Mendes, 1998).

In this study, we demonstrated that the possible roles of quercetin glucuronides on the human lung cancer NCI-H209 cells were 1) to decrease the percentage of viable cells in a dose- and time-dependent effect, 2) to arrest the cell cycle at the G2/M transition via cyclin B, WEEl, Cdc25C-ser-216-p, and p21 checkpoints, 3) to induce apoptosis via promoting the caspases-9 and -3 activities, and 4) to block apoptosis by cotreatment with the caspase inhibitor z-VAD-fmk. Our results provide evidence that quercetin glucuronide-induced apoptosis results from intrinsic pathway involved the mitochondrial cascades in NCI-H209 cells.

Although DNA damage due to reactive oxygen species (ROS) is involved in apoptosis, levels of hydrogen peroxide were not affected and the percentage of apoptosis was not influenced by cotreatment with catalase (the scavenger of H2O2) in NCI-H209 cells. This indicates that quercetin glucuronides may have antioxidant activity, and its induction of apoptosis is not dependent on the presence of H2O2, which is in agreement with the observation by Park et al. (2003).

It has been suggested that the intranuclear mechanisms after DNA damage are highly conserved in initiating apoptosis, cell cycle arrest, or DNA repair (Ferreira et al., 2002) The G2/M phase checkpoint plays a key role in providing time for DNA repair, and it provides an alternative pathway to apoptosis to remove irreparably damaged cells.
antiapoptotic members include Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl-1, and Bax/Diva, and a specific class of inhibitors of apoptosis proteins (IAPs) including c-IAP1, c-IAP2, XIAP, survivin, and others (Norbury and Zhivotovsky, 2004). Bax and Bak, normally located in the cytosol, are activated and then translocated to the mitochondria and permeabilize the outer mitochondrial membrane, facilitating release of cytochrome c from mitochondria to cytosol in response to apoptotic stimuli. These events subsequently trigger the formation of apoptosisosome, consisting of procaspase-9, Apaf-1, and cytochrome c in the presence of dATP, and turns on the executioner caspases-9 and -3 (Li et al., 1997; Green and Reed, 1998). Bax and Bak also act on the endoplasmic reticulum (ER), and their role on ER may be the release

FIG. 8. Representative Western blot showing changes in the levels of p21CIP1/WAF1, Bak, Bax, Bcl-2, cytochrome c, and caspase-3 in NCI-H209 cells after exposure to quercetin glucuronides. NCI-H209 cells (5 × 10⁶/ml) were treated with 0, 0.5, 1, 2.5, 5, and 10 μM quercetin glucuronides for 24 h, and then total proteins were prepared and determined as described under Materials and Methods. Then followed by evaluation of the levels of p21CIP1/WAF1, Bak, Bax, cytochrome c, Bcl-2, and caspase-3 expressions were estimated by Western blotting as described under Materials and Methods.
of Ca²⁺ ions and the activation of caspase-12, which subsequently activates the caspase-3 cascade (Li et al., 1997; Wei et al., 2001; Scorrano et al., 2003; Zong et al., 2003). Therefore, it is of great interest to further clarify the cross-talk between mitochondria and ER by quercetin glucuronides in cancer cells.

In conclusion, we demonstrated that quercetin glucuronides inhibited cell proliferation through cell cycle arrest at G1/M phase and induction of mitochondria-mediated apoptosis in human lung cancer cells (NCI-H209). Delineation of the biological effects of specific major quercetin metabolites on chemotherapeutic potential or chemoprevention of human cancers warrants further investigation.

References


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