Anticancer effect and apoptosis induction by quercetin in the human lung cancer cell line A-549

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Abstract. The aim of the present study was to investigate the anticancer effect of quercetin (QC) in the human lung cancer cell line A-549 and further study the mechanism of apoptosis induction by QC. Low differentiation potential A-549 human lung cancer cells were treated with QC at different doses and for different times, and the growth inhibitory rates were detected by MTT assay. Apoptosis induced by QC in A-549 cells was observed by Annexin V/PI double staining and flow cytometric assay. The relative tumor growth ratio of the treated/control tumors (T/C) (%) was chosen to represent the tumor growth inhibition of A-549 cell nude mouse xenografts by QC. Apoptosis of the nude mouse xenografts was observed by Annexin V/PI double staining and flow cytometric assay and DNA fragmentation assay. To further determine the molecular mechanism of apoptosis induced by QC, changes in the expression of bcl-2 and bax genes were detected by RT-PCR. Following incubation with QC, the cell growth of the low differentiation potential A-549 human lung cancer cells was dramatically inhibited in a dose-dependent manner. After the cells were exposed to QC for 24, 48 and 72 h, the IC50 value was 1.02±0.05, 1.41±0.20 and 1.14±0.19 µmol/l, respectively. Apoptosis in the A-549 cells induced by QC was noted. The apoptotic subpopulation of A-549 cells was approximately 12.96 and 24.58%, respectively, when cells were incubated with 1.2 µmol/l QC for 48 and 72 h. T/C (%) of A-549 nude mouse xenografts was 44.3, when the nude mice were treated with QC (8 mg/kg). Meanwhile, apoptosis induced by QC was observed in the A-549 nude mouse xenografts. Increased expression of the bax gene and decreased expression of the bcl-2 gene were noted using RT-PCR. Our results provide further evidence of the growth inhibition of the A-549 human lung adenocarcinoma cancer cell line by QC. This effect is associated with the induction of apoptosis in A-549 cells and the molecular mechanism may be related to the reduction in expression of the apoptosis-regulating gene bcl-2, and increase in expression of the apoptosis-regulating gene bax. These results were also confirmed in vivo.

Introduction

Quercetin (QC) (3,3’,4’,5,7-pentahydroxyflavone) (Fig. 1, R=OH) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. Frequently, QC occurs as glycosides (sugar derivatives); e.g., rutin (Fig. 1) in which the hydrogen of the R-4 hydroxyl group is replaced by a disaccharide.

QC is one of the most widely distributed bioflavonoids in the plant kingdom and is a common component of most edible fruits and vegetable. Humans consume approximately 1 g of dietary flavonoid daily. This compound has been shown to inhibit the growth of various human cancer cell lines (1-3), including leukemia, hepatocellular carcinoma, and estrogen receptor-positive breast carcinoma MCF-7 cells, suggesting that QC may have anticancer and anti-metastatic potential (4-6). The growth inhibitory effect of QC on tumor cells is found to be a consequence of its ability to interfere with the enzymatic processes involved in the regulation of cellular proliferation: DNA, RNA and protein biosynthesis (7,8). QC has also been shown to down-regulate or inhibit the phosphatidylinositol and phosphatidylinositol phosphate kinase activities in human carcinoma cells, leading to a marked reduction in second messenger IP3 concentration and cell death (9,10). Therefore, QC may be useful in the treatment of carcinomas with increased or down-regulated signal transduction capacity (10-12).

This study was undertaken to evaluate the growth inhibition and apoptosis induction of the human lung cancer line A-549 by QC, and the probable molecular mechanism.

Materials and methods

Materials. Medicine QC was supplied by the School of Pharmacy, Soochow University (norms, 20 mg/bottle). The sample was dissolved in RPMI-1640 medium (Gibco). Tumor A-549 cells supplied by the Cell Bank of Shanghai Institute of Cell Biology, were maintained in RPMI-1640 medium supplemented with 10% heated activated calf serum, benzylpenicillin 100 KIU/l, and streptomycin 100 mg/l, pH 7.4 in an incubator (Hirasawa, Japan) with a humidified atmosphere of 95% air + 50 ml/l CO2.
at 37°C. Athymic nude mice (BALB/c, nu/nu) (6-week-old) used for in vivo experiment, were obtained from the Shanghai Laboratory Animal Center (SLAC, China). The nude mice were handled using aseptic techniques and maintained in a specific pathogen-free environment on a 12 h light/12 h dark cycle, with food and water supplied ad libitum. The mice all weighed 18-22 g at the start of the study. Reagent for MTT purchased from Fluka, was dissolved in 0.01 mol/l PBS. PCR primers were synthesized by Sangon Shanghai.

Methods

Cell growth inhibition. Cells (2x10⁶/well) were seeded into each well of 96-well plates for 24 h, treated with various concentrations of QC (100 µl/well), and incubated for 24, 48, and 72 h, respectively. Then, 5 mg/ml MTT solution (20 µl/well) was added to each well, and cells were incubated for an additional 4 h at 37°C. The supernatant was aspirated, and 100 µl of DMSO was added to the wells to dissolve any precipitate present. The suspension was placed on a micro-vibrator for 5 min and the absorbance (A) was then measured at 570 nm by an enzyme immunoassay instrument. Cell inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = average absorbance of treated group/average absorbance of control group × 100%. IC₅₀ was calculated by SAS statistical software.

Assessment of apoptosis in A-549 cells by Annexin V/PI double-staining assay. Cells (1x10⁶) were seeded in 50-ml dishes and incubated for 24 h at 37°C. Then QC (1.2 µmol/l) was directly added to the dishes and incubated for an additional 24, 48, and 72 h, respectively. Cells were collected, washed with PBS and resuspended in PBS. Apoptotic cell death was identified by double supravital staining with recombinant FITC-conjugated Annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (Becton-Dickinson, USA) according to the manufacturer’s instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton-Dickinson FACSCalibur flow cytometer using CellQuest software.

Tumor growth inhibition of A-549 cell xenografts in nude mice by QC (9). A-549 cells (5x10⁶) were subcutaneously inoculated into BALB/cA nude mice. The transplanted tumors were ready to be used after 3 generations in nude mice. After 12-14 days, the nude mice with implanted tumor were screened for tumor growth. The mice all weighed twice per week, and mortality was monitored during the experimental period to assess toxicity of the treatments. Relative tumor volume (RTV) was calculated according to the equation: RTV = Vt/V0, where V0 is the tumor volume at day 0 and Vt is the tumor volume at day t. The evaluation index for inhibition of the relative tumor growth ratio was: T/C = TRTV/CRTV x 100%, where TRTV and CRTV represented RTV of the treated and control groups, respectively.

Apoptosis in the A-549 cell xenografts in the nude mice. Apoptosis in the human lung adenocarcinoma cancer line A-549 xenografts in nude mice was detected. BALB/cA nude mice were inoculated with A-549 cells, and fresh tumor tissue was collected after the mice were administered QC (8 mg/kg) i.v. 6 times. The positive control drug was 5-FU, with a dose of 25 mg/kg. Early apoptotic cell death was detected by Annexin V/PI double-staining assay, using the Annexin V-FITC Apoptosis Detection kit. DNA fragmentation assay was used to assess late apoptosis induced by QC in the A-549 nude mouse xenografts. In this assay, DNA was extracted by Genomic DNA Purification kit (Fermentas), and then the products were electrophoresed on a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer.

RT-PCR. Cells (1x10⁶) were treated in the presence or absence of 0.8 µmol/l QC for 24, 48 and 72 h, respectively, and total RNA was extracted by TriPure isolation reagent (Roche). The primers for Bcl-2, Bax and GADPH were as follows: bcl-2 (340 bp), 5'-TTCCCATCGCTGTCTTTCCG-3', 3'-CGTCTTAGATACAAATTGTCCGTGTC-5'; bax (386 bp), 5'-GGATGCGTCACCAAGAA-3', 3'-AAACACGCGCTTACAG-5'; GADPH (450 bp): 5'-CTCAGACACATGGGAGAGAAGTGA-3', 3'-ATACTGTGTGGAGAGTGTTAGTA-5'. The following PCR conditions were used, 94°C for 5 min, 1 cycle; 94°C for 30 sec, 59°C (bcl-2) or 56°C (bax) for 30 sec, 72°C for 45 sec, 35°C (bcl-2) or 30°C (bax) 1 cycle; 72°C for 7 min, 1 cycle. The PCR products were electrophoresed on a 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer.

Results

Cell growth inhibition. A time- and concentration-dependent growth inhibition was observed in the low differentiation potential human lung cancer cell line A-549 after treatment with QC for 24, 48 and 72 h. The IC₅₀ values at 24, 48, and
72 h were 2.30±0.26, 1.41±0.15, and 1.02±0.15 µmol/l, respectively. This indicated that when the concentration of QC was <0.74 µmol/l, the inhibition was weak. In addition, when the concentration ranged from 0.74 to 4.40 µmol/l, the inhibitory effect became obvious; the higher the concentration, the stronger the effect. When the concentration was >4.40 µmol/l, the inhibitory effect was no longer changed. Data of 3 experiments are shown as the mean ± SD (Fig. 2).

Assessment of apoptosis in A-549 cells by Annexin V/PI double-staining assay. The results of Annexin V/PI double-staining assay demonstrated that the apoptosis of A-549 cells was observed after treatment with 1.2 µmol/l QC for 48 and 72 h, respectively. As shown in Fig. 2, untreated cells did not show any significant apoptosis, as well as cells treated with 1.2 µmol/l QC for 24 h, whereas cells became apoptotic after treatment with 1.2 µmol/l QC for 48 and 72 h, with apoptotic populations of ~12.96 and 24.58%, respectively (Fig. 3).

Tumor growth inhibition of A-549 cell xenografts in nude mice by QC.

The results showed that QC, as well as 5-FU, inhibited the growth of A-549 nude mouse xenografts. The therapeutic effect was optimal on the 12th day after the drug was administered to the tested groups first. RTV of the test group treated with 8 mg/kg QC was 5.2 and T/C (%) was 44.3; RTV of the group treated with 4 mg/kg QC was 8.4 and T/C 824-826, 2012

Table I. Inhibitory growth effect of QC in A-549 cell tumor xenografts.

<table>
<thead>
<tr>
<th>QC concentration (µmol/l)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
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<tbody>
<tr>
<td>0.52</td>
<td>2.37±0.45</td>
<td>3.88±1.05</td>
<td>13.43±2.46</td>
</tr>
<tr>
<td>0.74</td>
<td>5.51±0.63</td>
<td>10.11±1.89</td>
<td>30.05±2.44</td>
</tr>
<tr>
<td>1.06</td>
<td>14.50±3.12</td>
<td>30.84±4.77</td>
<td>53.25±4.65</td>
</tr>
<tr>
<td>1.51</td>
<td>32.71±4.23</td>
<td>58.49±6.24</td>
<td>71.88±3.91</td>
</tr>
<tr>
<td>2.16</td>
<td>55.73±4.38</td>
<td>78.56±7.18</td>
<td>86.57±4.42</td>
</tr>
<tr>
<td>3.08</td>
<td>70.44±7.38</td>
<td>91.67±5.38</td>
<td>94.89±3.80</td>
</tr>
<tr>
<td>4.40</td>
<td>81.83±5.08</td>
<td>97.80±2.52</td>
<td>99.07±0.29</td>
</tr>
<tr>
<td>6.29</td>
<td>89.53±5.71</td>
<td>97.67±1.94</td>
<td>98.54±0.96</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>2.30±0.26</td>
<td>1.41±0.15</td>
<td>1.02±0.05</td>
</tr>
</tbody>
</table>

Table II. Growth inhibition by QC in transplanted tumors induced by A-549 cells in BALB/cA nude mice (control, n=12; QC and 5-FU, n=6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor weight (g)</th>
<th>Inhibiting ratio of growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-549 (control)</td>
<td>3.616±0.718</td>
<td></td>
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<tr>
<td>QC (4 mg/Kg)</td>
<td>3.220±0.845</td>
<td>19.24</td>
</tr>
<tr>
<td>QC (8 mg/Kg)</td>
<td>2.425±0.920b</td>
<td>44.30</td>
</tr>
<tr>
<td>QC (8.4 mg/Kg)</td>
<td>2.096±0.265c</td>
<td>71.10c</td>
</tr>
<tr>
<td>5-Fu (25 mg/kg)</td>
<td>2.120±0.316d</td>
<td>70.04d</td>
</tr>
</tbody>
</table>

a vs b, a vs c, a vs d, p<0.05.

Assessment of apoptosis in A-549 cells by Annexin V/PI double-staining assay. The results of Annexin V/PI double-staining assay demonstrated that the apoptosis of A-549 cells was observed after treatment with 1.2 µmol/l QC for 48 and 72 h, respectively. As shown in Fig. 2, untreated cells did not show any significant apoptosis, as well as cells treated with 1.2 µmol/l QC for 24 h, whereas cells became apoptotic after treatment with 1.2 µmol/l QC for 48 and 72 h, with apoptotic populations of ~12.96 and 24.58%, respectively (Fig. 3).

Tumour growth inhibition of A-549 cell xenografts in nude mice by QC. The results showed that QC, as well as 5-FU, inhibited the growth of A-549 nude mouse xenografts. The therapeutic effect was optimal on the 12th day after the drug was administered to the tested groups first. RTV of the test group treated with 8 mg/kg QC was 5.2 and T/C (%) was 44.3; RTV of the group treated with 4 mg/kg QC was 8.4 and T/C
This suggests that QC (8.4 mg/kg) distinctly inhibits the growth of transplanted tumors induced by A-549 cells in BALB/cA nude mice (P<0.05) (Table I and II).

Apoptosis in A-549 nude mouse xenografts. Annexin V/PI staining assay and DNA fragmentation assay were used to detect the apoptosis induced by QC in A-549 nude mouse xenografts. Results of the Annexin V/PI staining assay showed that the early apoptotic population was ~25% after treatment with QC (8 mg/kg) (Fig. 5). At the same time, DNA fragmentation assay provided evidence of DNA fragmentation and morphological changes in nuclei in the A-549 cells treated with QC (Fig. 5).

Expression of the bcl-2 and bax genes. It was confirmed that expression of the bcl-2 and bax genes was regulated by QC. As shown in Fig. 7A-C, after incubation with 0.8 µmol/l QC for 24, 48 and 72 h respectively, the expression of bcl-2 mRNA decreased dramatically, while, on the contrary, the expression of bax mRNA increased. In addition, the ratio of bax/bcl-2 increased (13,14) (Fig. 6D). The results demonstrated that QC down-regulated the expression of bcl-2 gene and up-regulated the expression of the bax gene. When the cells were treated with QC (0.8 µmol/l) for 48 h, the regulation was the most distinct.

Discussion

QC is a flavonoid molecule ubiquitous in nature. In recent years, research involving QC has ranged from considering it as a potentially carcinogenic agent to examination of its promise as an anticancer agent. Four pressing questions require elucidation. Is additional dietary supplementation safe? Is QC absorbed and bioavailable when administered orally? Is it active against malignant human cells and could its use be developed? Are additional routes such as intravenous or transdermal safe or more advantageous? It is the object of this review to present evidence concerning these issues and outline gaps in the available data which need to be filled in order to determine whether QC has an appreciable role in future cancer therapy.

In this study, we analyzed the effects of QC on the proliferation and apoptosis of a human lung cancer cell line A-549 in vitro and in vivo. The dramatic growth inhibition of QC in A-549 cells was observed by MTT assay and the greatest inhibitory rate was 99.07%. Further experiments demonstrated that the cell death was partly caused by the apoptosis induced by QC. In addition, we obtained the same results in the in vivo experiments. Result of the growth inhibition assay of the A-549 nude mouse xenografts demonstrated that the inhibitory effect of QC (8 mg/kg), as well as 5-FU, was dramatically greater than that in the negative control. Apoptosis in the A-549 cell nude mouse xenografts was detected and the apoptotic population of tumor cells reached 25%; the ‘DNA ladder’ was obvious. Thus, the results confirmed that QC strongly inhibited the proliferation of human lung cancer cell line A-549 in vitro.
and in vivo by inducing apoptosis. To further discuss the exact mechanism of apoptosis induced by QC, we assessed changes in expression of the bcl-2 and bax genes by RT-PCR. The results showed that QC down-regulated the expression of bcl-2 mRNA and up-regulated the expression of bax mRNA. This confirmed that the inhibitory effect of QC was associated with an induction of apoptosis through the regulation of expression of the bcl-2 and bax genes. Apoptosis represents a major protective mechanism against cancer (15), and our observation demonstrated that QC induced apoptosis in A-549 cells. Thus, we deduced that the high growth inhibition by QC in A-549 cells was partly caused by its induction of apoptosis. To further discuss the exact molecular mechanism of apoptosis induced by QC, expression levels of the apoptosis-regulating gene bcl-2 and bax were assessed. It was previously confirmed that the bcl-2 gene acts to inhibit apoptosis (16,17), while the bax gene induces apoptosis (18,19). Our results from RT-PCR showed that after treatment with QC (0.8 µmol/l) for 48 h, bcl-2 expression was dramatically decreased and bax expression was slightly increased at the mRNA level and the ratio of bax/bcl-2 increased. Thus, apoptosis occurred (20). We conclude that QC induces apoptosis by up-regulating expression of the bax gene and down-regulating expression of the bcl-2 gene. Moreover, we suggest that the up-regulated expression of the bax gene induced by QC led to an increase in the expression of bax, and the latter not only inhibited the function of bcl-2 in apoptosis by forming heterodimers (14), but also increased the release of cytochrome C (21,22) from the inter-membrane space to the cytosol, and thus the ‘intrinsic’ pathway (23-26) of apoptosis was activated. In summary, the anticancer effect of QC may be connected with its ability to regulate the expression of apoptotic-related genes.

Acknowledgements

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References