Quercetin induces apoptosis by activating caspase-3 and regulating Bcl-2 and cyclooxygenase-2 pathways in human HL-60 cells

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Quercetin is one of the naturally occurring dietary flavonol compounds. It is present abundantly in plants and has chemopreventive and anticancer effects. To investigate its anticancer mechanism, we examined the activity of quercetin against acute leukemia cell line, HL-60. Our results showed that quercetin inhibited cell proliferation and induced apoptosis in a time- and dose-dependent manner. Furthermore, quercetin down-regulated the expression of anti-apoptosis protein Bcl-2 and up-regulated the expression of pro-apoptosis protein Bax. Caspase-3 was also activated by quercetin, which started a caspase-3-dependent mitochondrial pathway to induce apoptosis. It was also found that quercetin inhibited the expression of the cyclooxygenase-2 (Cox-2) mRNA and Cox-2 protein. Taken together, these findings suggested that quercetin induces apoptosis in a caspase-3-dependent pathway by inhibiting Cox-2 expression and regulates the expression of downstream apoptotic components, including Bcl-2 and Bax. Quercetin can be a potent and promising medicine which might be safely used in leukemia therapy.

Keywords quercetin; apoptosis; cyclooxygenase-2; leukemia

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Introduction

Acute leukemia is a common malignancy of the hematopoietic progenitor cell and has increased incidences in recent years. Significant progress has been made in chemotherapy treatment of acute leukemia, but some patients failed to get complete remission due to drug resistance [1,2]. Therefore, it is important to seek some novel drugs to increase the survival rate of acute leukemia patients. Vegetables, fruits and grains may offer significant protection against various cancers, including leukemia. Quercetin, one of several naturally occurring dietary flavonol compounds, is present abundantly in fruits, vegetables and seeds and exhibits a wide range of pharmacological effects, including antioxidant, anti-inflammatory, anti-anaphylaxis, anti-platelet, neuroprotection and so on [3,4]. Recent studies have shown that quercetin has anti-tumor effects on several malignant leukemia cell lines such as HL-60, K562, NB4 and Jurkat and also is considered as a potential anti-cancer drug for clinical use [5–9].

The exact molecular mechanisms underlying its anti-cancer effects associated with quercetin have not been fully explained yet. Quercetin has been shown to inhibit cell proliferation, induce apoptosis and antioxidant. Quercetin can modulate a number of key targets in cellular signal transduction pathways related to the apoptosis and cell proliferation such as downstream anti-apoptosis proteins of the Bcl-2 family (Bcl-XL and Bcl-2) and up-regulating pro-apoptotic members, Bax and Bad [8,10]. It has also been demonstrated that quercetin induces apoptosis through a caspase-3- and caspase-9-dependent mechanism by releasing cytochrome c and PARP (poly-(ADP-ribose) polymerase) cleavage [11,12]. In addition, quercetin can inhibit the proteasome activity through modulation of signaling components such as serine/threonine kinase, Akt/protein kinase B, phosphatidylinositol-3 kinase (PI-3-kinase) and extracellular-regulated kinase [8,13,14]. Quercetin not only inhibits tumor cells but also may reverse the resistance of tumor cells [15].

Cyclooxygenases (Coxs) are known as prostaglandin H synthases, which convert arachidonic acid released by membrane phospholipids into prostaglandins. Two isoforms of cyclooxygenases, Cox-1 and Cox-2, have been identified. Cox-1 is constitutively expressed in many tissues, whereas Cox-2 is only over-expressed in practically premalignant and malignant condition. Quercetin as an anti-inflammatory drug can inhibit Cox-2 through reducing PGE2 expression [16,17]. Some studies also demonstrated that quercetin can induce apoptosis through suppressing Cox-2 in in vivo and in vitro models [18–20]. Cox-2 was found to be over-expressed in leukemia. Some specific
Cox-2 inhibitors, such as celecoxib, can suppress cell proliferation and induce leukemia cell apoptosis [21–23]. Whether quercetin can inhibit the proliferation of leukemia through suppressing Cox-2 is still unknown. In this study, we observed that quercetin could induce apoptosis in human leukemia HL-60 cells. To understand the molecular mechanism under its cellular effects, we explored the roles of Cox-2 and other signaling components in the induction of apoptosis.

Materials and Methods

Materials
Quercetin was purchased from Sigma Chemical Company (St. Louis, USA). It was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mM and stored at −20°C before use, and diluted to different concentrations with RPMI-1640 medium for assays. The final concentration of DMSO was maintained at 0.1%. Mouse monoclonal anti-human Cox-2 antibody was purchased from Cayman Chemical Company (Ann Arbor, USA) and mouse monoclonal anti-human Bcl-2 antibody was obtained from R&D systems (Minneapolis, USA). Rabbit monoclonal anti-human Bax and mouse monoclonal anti-human caspase-3 antibodies were provided by Cell Signaling Technology (Beverly, USA).

Cell line and cell culture
Human leukemia cell line HL-60 was purchased from Pharmacology Department of Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College (Tianjin, China). The cells were maintained in RPMI-1640 medium (Gibcol, Grand Island, USA) containing 10% fetal bovine serum (Sijiqing, Hangzhou, China) in an incubator at 37°C with a humidified atmosphere containing 5% CO₂.

Cell counting kit-8 (CCK-8) assay for cell viability
The CCK-8 assay was used to determine the effects of quercetin on proliferation of leukemia HL-60 cells. Briefly, exponentially growing cells were diluted to a concentration of 5 × 10⁴ cells/ml in RPMI-1640 medium, plated in 96-well plates (Corning Inc., Corning, USA) with 100 μl/well, followed by the addition of quercetin at varying concentrations (12.5–100 μM) and incubated at 37°C for 24, 48 or 72 h. At the end of each time point, 10 μl of CCK-8 solution was added to the wells and incubated for 4 h. Finally, the plates were read in an enzyme-linked immunity implement (Wellskan MK3; Labsystem Dragon, Helsinki, Finland) at 450 nm with a reference wavelength of 630 nm. All samples were assayed in triplicate in three independent experiments. The calculation formula of CCK-8 assay is as follows: cell viability = experiment group OD/control group OD.

Cell apoptosis by flow cytometry
After treatment with varying concentrations of quercetin for 48 h, the cells were washed twice with phosphate-buffered saline (PBS) and diluted to a final concentration of 1 × 10⁶ cells/ml, then incubated with Annexin V-FITC and PI for 30 min without light. The cell apoptosis was determined by flow cytometry (FACS Becton-Deckinson, Temse, Belgium). Early stage of cell apoptosis was defined in Annexin V-FITC(+)/PI(−) and late stage was Annexin V-FITC(+)PI(+).

Detection of apoptosis by Hoechst 33342 chromatin staining
The cells treated with quercetin for 48 h were collected by centrifugation at 250g, for 5 min and fixed with 3.7% paraformaldehyde for 20 min, then painted on the slides and stained with 10 μM Hoechst 33342 dye (Sigma) for 10 min. After washing with PBS, the fluorescence intensity was assessed using a fluorescence microscope (Lecia, Bensheim, Germany).

Western blot analysis
The cells were incubated with quercetin at the concentration of 0, 25, 50 and 100 μM for 48 h, then washed with cold PBS and kept on ice with radio immunoprecipitation assay (RIPA) lysis buffer (Biyuntian, Shanghai, China) containing 1% phenylmethylsulfonyl. The cell debris was collected 30 min later by centrifugation at 13,400 rpm for 30 min at 4°C. The protein contents were measured by Bradford method (Shennengbocai, Shanghai, China). The samples were loaded on SDS–PAGE gel for electrophoresis analysis and then transferred onto polyvinylidene fluoride membrane. After incubation overnight at 4°C with the specific monoclonal antibody mentioned above, horseradish peroxidase-linked secondary antibody (Boster, Wuhan, China) was used, and the protein band was detected using DAB kit (Pulilai, Beijing, China) following the manufacturer’s instruction.

RT–PCR and quantitative RT–PCR for Cox-2 mRNA
The cells treated with quercetin for 48 h were collected to extract cellular RNA using RNAiso reagent (TaKaRa, Shiga, Japan). Briefly, total RNA isolated was used for first-strand cDNA reverse transcription by using cDNA synthesis kit (TaKaRa) according to manufacture’s instruction. The cDNA of Cox-2 was amplified with the gene-specific primers and the first strand of cDNA synthesized previously with the following program: 35 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 1 min; followed by a 10 min extension. The products were subject to electrophoresis on a 2% agarose gel and then detected under UV.
light. The following primers were used for Cox-2 amplification: 5'-ATCCTAGTCTCTACGTGCCC-3' (forward), 5'-TTGTCACAGGAGCTGCTG-3' (reverse). The predicted size of the fragment was 301 bp. β-Actin was amplified as a control gene for Cox-2 with a set of gene-specific primers, including 5'-CTACAATGAGCTGCGTGTGG-3' (forward) and 5'-AAGGAAGGCTGGAAGAGTGC-3' (reverse) primers.

To detect Cox-2 expression, quantitative RT–PCR was performed with the cDNA products mentioned above using SYBR green PCR master reagents (TaKaRa). β-Actin was used as a control and each experiment was repeated three times. The gene-specific primers used in RT–PCR were as follows: Cox-2 5'-GCTGGAACATGGAATTACCCAGTT-3' (forward), 5'-GAGTGCTTCCAACTCTGCAGACA-3' (reverse); β-actin 5'-TTTCTTCTGOGCATGGAGTCC-3' (forward), 5'-GGAGGAGGCAAAGGCTGCTTGC-3'. Amplification was carried out with an initial incubation at 94 °C for 4 min, followed by 94 °C for 15 s, 58 °C for 15 s and 72 °C for 15 s, for a total of 40 cycles. Data analysis was completed using the 7500 Sequence Detection software and the 2^−ΔΔCt method for relative quantization.

Statistical analysis
All experiments were carried out in triplicate. Data were presented as mean ± SD. Student’s t-test was used in comparison between the two groups, and one-way ANOVA was used in multiple comparisons with SPSS 13.0 software (SPSS, Chicago, IL, USA). P < 0.05 was considered statistically significant. All P-values are represented as two-sided.

Results
The proliferation effects of quercetin on HL-60 cells
CCK-8 assay was used to detect cell viability after treatments and the results showed that quercetin inhibited proliferation of HL-60 cells in a dose- and time-dependent manner. After treatment with 12.5–100 μM quercetin for 24 h, cell viability was still >80% and not significantly different with that of control, indicating that quercetin showed slight cell toxicity in these dosages. However, when incubation time extended to 48 h, quercetin at the same concentrations (12.5–100 μM) significantly inhibited cell proliferation [Fig. 1(A)]. The calculated cell inhibitory concentration 50 (IC50) 48 h later was 61.11 μM. When incubation time extended to 72 h, the cell viability decreased slightly when compared with that at 48 h. Therefore, we choose cell culture for 48 h for following experiments. Meanwhile, cells without treatment were observed to be normal with round or oval clear nuclei under microscope. After quercetin treatment, cells were irregular and shrunken, and part of cytoplasm contained hollow bubbles. When concentration increased and incubation time extended, the cell morphology change became apparent with more broken cells, more cellular contents released and cellular debris increased. The morphology change also showed that HL-60 cell vitality gradually decreased with an increase in quercetin concentration [Fig. 1(B)].

Quercetin-induced HL-60 cell apoptosis
The cells were treated with quercetin (0, 25, 50 and 100 μM) for 48 h and the apoptosis rate was then calculated by flow cytometry. The average of apoptosis rate at early stage was 2.4, 8.2, 15.06 and 19.29% when treated with quercetin at 0, 25, 50 and 100 μM, respectively (Fig. 2). The P-value calculated was <0.05 between control group (0 μM) and the group treated with 25 μM quercetin, and was <0.01 between the control group and the other two treatment groups (50 and 100 μM), suggesting that quercetin significantly suppressed cell growth by inducing apoptosis. These results were

Figure 1 Quercetin suppressed HL-60 cell growth (A) The inhibitory effects of quercetin on HL-60 cells were evaluated by CCK-8 assay. The HL-60 cells were seeded in 96-well plates and incubated with quercetin for 24, 48 and 72 h at the concentration of 0, 12.5, 25, 50, 75 and 100 μM, respectively. *P < 0.05 and **P < 0.01 compared with the control. (B) The morphology change of cells treated with quercetin was detected under microscope. The picture of number 1, 2, 3 and 4 represents the morphology of HL-60 cells treated with quercetin at the concentration of 0, 25, 50 and 100 μM for 48 h, respectively.
consistent with those of morphology change of cells stained with Hoechst 33342 fluorescent dye (Fig. 3). The normal HL-60 cells [Fig. 3(A)] had clear nuclei with pale blue staining, while the nuclei of cells treated with quercetin had a condensed and cleaved morphology [Fig. 3(B)].

**Effects of quercetin on Bcl-2/Bax, caspase-3 and Cox-2 protein expression**

After cells were exposed to quercetin (0, 25, 50 and 100 μM) for 48 h, Cox-2 and Bcl-2 protein levels were significantly decreased, while Bax protein level was significantly increased [Fig. 4(A)]. After treatment with 50 μM quercetin for 48 h, the Cox-2 and Bcl-2 protein levels were decreased 1.4- and 5.7-folds, respectively, while the Bax level increased 2.9-fold. Meanwhile, caspase-3 was up-regulated 1.1-fold and the cleaved product of caspase-3 was increased in a dose-dependent manner [Fig. 4(B)]. The change in Bcl-2/Bax and caspase-3 protein level indicated that quercetin could induce apoptosis through a mitochondria-mediated mechanism. Cox-2 expression was inhibited by quercetin in a dose-dependent manner, which was consistent with quercetin-regulated expression of other apoptotic proteins. It indicated that apoptosis induced by quercetin was initiated by inhibition of Cox-2 protein which might regulate Bcl-2/Bax expression to activate a caspase-3-dependent pathway.

*Figure 2 Apoptosis induced by quercetin in HL-60 cells*  
The cells were treated with quercetin at the concentration of 0, 25, 50 and 100 μM, respectively, for 48 h. The apoptosis rate was calculated by flow cytometry. Cell apoptosis at early stage was defined in Annexin V-FITC(+)PI(-). *P < 0.05 and **P < 0.01 compared with the control.
Quercetin inhibits Cox-2 expression in HL-60 Cell

To investigate whether the decreased Cox-2 protein level shown above is due to decreased Cox-2 transcription, RT-PCR and quantity RT-PCR assays were performed. The cells were treated with quercetin at the concentration of 0, 25, 50 or 100 μM for 48 h and then used for RNA isolation. The RT-PCR gel analysis showed that the expression of Cox-2 gene decreased significantly compared with the control [Fig. 5(A)], which was consistent with the result of quantity RT-PCR: the expression of Cox-2 gene significantly decreased 1.50-, 4.60- and 5.14-fold compared with that of internal control, actin, respectively. [Fig. 5(B)].

Discussion

Intake of some plant foods such as grapes and vegetables has been known as prevention of cancer and cardiovascular diseases for a long time [24,25]. Flavonoids in these plants have been considered to play an important role in prevention of these diseases. Quercetin, as one of important flavonoid compounds, has been frequently studied for its anti-cancer effects. The present studies have showed that quercetin could inhibit growth of some malignant leukemia cell lines, but the molecular mechanism is still unclear. In this study, we demonstrated that quercetin could induce apoptosis in human HL-60 cells.
apoptosis in HL-60 leukemia cell line through activation of a caspase-3-dependent pathway which might be initiated by inhibition of Cox-2 expression.

We first evaluated the proliferation inhibitory effect of quercetin in HL-60 cells. Chen et al. showed that quercetin at 50 μM treatment for 24 h resulted in 70% non-viable cells in Jurkat T-cell line [8]. Our data showed that the inhibition effect was not obvious after 24-h treatment with quercetin (12.5–100 μM) in HL-60 leukemia cell line. When treatment time extended to 48 h and even 72 h, the inhibition rate increased significantly. Quercetin could also significantly inhibit cell growth in a time- and dose-dependent manner. The toxicity data as well as morphological changes in this paper clearly demonstrated that quercetin could efficiently inhibit growth of leukemia cells.

To explore the possible mechanisms of the inhibitory effects of quercetin on the proliferation of HL-60 cells, the assays of Hoechst staining and flow cytometry were carried out to detect apoptosis. When HL-60 cells were treated with quercetin for 48 h at the concentration of 0, 25, 50 and 100 μM, the result of Hoechst staining clearly showed apoptotic bodies under fluorescence microscope; FACS assays indicate that the percentage of apoptosis cells were enhanced with an increase of concentrations. At the concentration of 100 μM quercetin, the percentage of apoptosis cells at early stage was 19.29(±0.91)% and necrotic cells at late apoptosis 40.68(±4.85)% (data not shown). These results above clearly showed that quercetin induced cell apoptosis which might lead to inhibition of proliferation.

Previous studies have shown that quercetin could suppress the synthesis of the Bcl-2 protein and the Bcl-2 mRNA level in different malignant tumor cell lines, including acute leukemia cell line HL-60 and chronic B-cell leukemia lines [26,27]. Results in this study are consistent with previous research that the Bcl-2 protein level decreased when treated with quercetin at low dose or high dose. The low dose of 25 μM significantly decreased Bcl-2 protein 1.4-folds and the high dose of 100 μM could even inhibit Bcl-2 expression 27-folds. In this study, data also showed the pro-apoptosis proteins such as Bax and caspase-3 increased in a dose-dependent manner. Similar results were obtained by Chen et al. in leukemia Jurkat T cells [8]. All these findings suggested that apoptosis of acute leukemia cells caused by quercetin might be induced by activation of a caspase-3-dependent mitochondrial pathway.

We further studied an apoptotic upstream regulatory gene, Cox-2. Cox-2 has been known to play a critical role in leukemia development [28,29], and the inhibition of Cox-2 may become an alternative method to treat or prevent leukemia. Lee et al. found that quercetin inhibited the expression of Cox-2 to induce apoptosis in both breast cancer and colon cancer cells [30]. In this study, we observed that quercetin could also inhibit the expression of Cox-2 in both protein and mRNA level in HL-60 cells. Tsuji and DuBois found in 1995 that an artificial increase of the expression of Cox-2 in epithelial cells can induce the expression of Bcl-2 protein to enhance cell resistance to apoptosis [31]. Later, a series of studies have shown that Cox-2 inhibited the apoptosis of tumor cells by increasing anti-apoptosis protein Bcl-2 [32,33]. However, Hsu et al. found that celecoxib as a Cox-2 inhibitor induced apoptosis in a Bcl-2-independent way [34]. This study showed that the Bcl-2 protein level decreased significantly after quercetin treatment in HL-60 cells. Together with other results, we proposed that the inhibition of Cox-2 expression by quercetin might reduce the expression of Bcl-2 to activate caspase-3 and trigger a caspase-3-dependent pathway to induce apoptosis.

This study demonstrated that the inhibition of HL-60 cell growth caused by quercetin might be due to inhibition of Cox-2 expression. Quercetin might reduce the expression of Cox-2 in a Bcl-2-dependent way and then activate caspase-3 to induce apoptosis in HL-60 cell. Quercetin is also known to regulate many signal transduction pathways such as AMP kinase, Akt kinase and so on, which may be associated the expression of Cox-2. Our research in the future will focus on how quercetin-signaling pathways regulate the expression of Cox-2.
Quercetin has also been reported to have its anti-tumor effects against a variety of tumors in vivo experiments. In rats, quercetin could reduce the growth of diethylnitrosamine-induced hepatocarcinoma when quercetin was introduced intravenously to rats (8.98 mM/kg) once a week for 16 weeks [35]. However, quercetin may exacerbate the development of estrogen-induced breast tumors. Breast tumors were developed in female August-Copenhagen Irish (ACI) rats by given quercetin (2.5 g/kg food) for 8 months in combination with treatment of estradiol, a powerful female hormone. Further experiments showed that this might be due to inhibiting the metabolism of estradiol by quercetin in rats [36]. In 1996, David et al. [37] have performed Phase I clinical trial with quercetin in humans. They found that quercetin with bolus injection could not only significantly decrease tumor cells in patient with refractory ovarian cancer, but also possibly caused nephrotoxicity at the high dose of quercetin. However, our study showed that only quercetin at a relatively high dose could inhibit the growth of leukemia cells, which might limit its clinical application. It is important to find some new strategies such as combination of other drugs and application of medicines in alternate ways in clinical trials.

Quercetin, which can target multiple anti-tumor proteins, has been demonstrated in this study to have effects on Cox-2 expression in HL-60 cells at the first time. This result has greatly extended our knowledge about the underlying molecular mechanism. With more understanding of its mechanism, quercetin can be a potent and promising medicine which may be safely used in leukemia therapy.

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