Resveratrol and quercetin-induced apoptosis of human 232B4 chronic lymphocytic leukemia cells by activation of caspase-3 and cell cycle arrest

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Chronic lymphocytic leukemia (CLL), defined by accumulation of pathogenic B cells, has a very complex biology due to various factors such as inherited, host, and environmental factors. Recently, finding new therapeutic agents or development of novel treatment strategies have been paid attention. Resveratrol and quercetin, important phytoalexins found in many plants, have been reported to have cytotoxic effects on various types of cancer. In this study, we examined cytotoxic, cytostatic, and apoptotic effects of these two important phenolic compounds on 232B4 human CLL cells. Cytotoxic effects of resveratrol and quercetin were determined by MTT cell proliferation assay. Changes in caspase-3 enzyme activity were measured using caspase-3 colorimetric assay. Annexin V-FITC/PI double staining was performed to measure apoptotic cell population. Effects of resveratrol and quercetin on cell cycle profiles of CLL cells were investigated by flow cytometry. Treatment of CLL cells with resveratrol and quercetin caused dose dependent inhibition of cell proliferation and increased apoptotic cell population through induction of caspase-3 activity. Cell cycle analysis displayed cell cycle arrest mainly in G0/G1 for both polyphenols. Our data, in total, showed for the first time that resveratrol and quercetin might block CLL growth through inducing apoptosis and cell cycle arrest.

Keywords: Chronic lymphocytic leukemia, Resveratrol, Quercetin, Apoptosis

Introduction

Chronic lymphocytic leukemia (CLL), the most common type of leukemia in the Western world, affects mainly adults.¹ CLL cells are originated from antigen-stimulated mature B lymphocytes that either avoid death by external signals or die by apoptosis. Therefore, CLL is characterized by the clonal proliferation and accumulation of antigen experienced B lymphocytes in the blood, bone marrow, lymph nodes, and spleen.²,³ Several genetic aberrations have been identified in CLL. The most common one is a deletion at 13q14.3 (IGHV). The other mutations include 11q22–23 (ATM), 17p13 (p53), and 6q21 deletions.⁴ These abnormalities are responsible for the formation of CLL and some of its clinical outcomes.

Although there are many therapeutic regimens, CLL is still an incurable disease.¹ Current treatment possibilities include conventional chemotherapy, monoclonal antibodies and hematopoietic transplantation.⁵ However, these standart treatment methods are not sufficient to remove all CLL cells and have their own side effects. Therefore, developing new strategies or agents that could eliminate CLL cells, resistant to apoptosis, is urgently required. Recently, there is an increasing interest in the usage of agents derived from natural products for cancer therapy. Plant products with strong antioxidant activities are called polyphenols and their effects on cell proliferation, gene regulation and apoptosis have been studied on several cancer cells.⁶

Resveratrol (trans-3,4',5-trihydroxystilbene), a kind of naturally occurring polyphenolic compound, is produced in a wide variety of plants like the skin of red grapes, peanuts, and mulberries to protect them against pathogenic attacks, injury and UV radiation.⁷,⁸ It is very well known that resveratrol shows antioxidant, cardioprotective, chemopreventive, anticarcinogenic, antiproliferative and anti-inflammatory effects both in vivo and in vitro.⁹,¹⁰ Resveratrol has been
shown to induce apoptosis in a variety of cancers such as chronic myeloid leukemia (CML), acute myeloid leukemia (AML), breast, prostate, colon and lung cancers. The exact mechanism of resveratrol mediated anticancer effect is not fully identified. As indicated in several studies, resveratrol-induced apoptotic mechanisms include inhibition of retinoblastoma (Rb) phosphorylation, suppression of NF-κB and STAT3, induction of p21 and 21,14 and ceramide synthases and inhibition of sphingosine kinase-1 and glucosyle ceramide synthase.12,13 It has been also shown that resveratrol inhibits the cell cycle progression by causing arrest at G1/G2 phases.15

Quercetin, an abundant polyphenol of the flavonoid family, is generated in onions, apples, red grapes, and other fruits and vegetables. The study of quercetin as a potential anticancerogen has been paid attention due to its high toxicity on cancer cells compared with its relatively scarce effects on normal cells. The antican
cerogenic effects of quercetin on numerous cancer cells including melanoma cells, colon, breast, endo
tetrial, T-cell acute lymphoblastic leukemia (ALL), acute promyelocytic leukemia, and chronic myeloid leukemia (CML) cells has been described.17

Mechanisms of quercetin triggered apoptosis include activation of caspases, upregulation of Bax and Bak and downregulation of Bel-2 and Bel-xL and inhibition of telomerase enzyme activity.18,20 Moreover, quercetin inhibits PI3 K-Akt/PKB pathway responsible for cell survival and proliferation. In some cancer cell types, quercetin induces apoptosis by upregulating death receptor-5.21

In this study, we have shown that resveratrol and quercetin, most abundant polyphenols, have cytotoxic and apoptotic effects on human 232B4 CLL cells. These two polyphenols induce apoptosis through loss of mitochondrial membrane potential and increases in caspase-3 enzyme activity.

Materials & methods

Chemicals

Resveratrol was obtained from Sigma-Aldrich (MO, USA). Resveratrol was dissolved in dimethylsulfoxide (DMSO) at a final concentration of 10 mM, stored at −20°C. Quercetin was obtained from Prof. Dr. Güray Saydam from Ege University, Department of Hematology. 10 mM stock solution of quercetin was prepared in DMSO and stored at −20°C. The final concentration of DMSO did not exceed more than 0.1% in culture.

Cell lines and culture conditions

Human 232B4 CLL cells were kindly provided by Prof. Dr. Ander Rosen from Linköping University. The cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in 5% CO2.

Measurement of cell growth by MTT cell proliferation assay

The IC50 values (drug concentration that inhibits cell proliferation by 50%) of resveratrol and quercetin were determined from cell proliferation plots obtained by MTT assay. Briefly, 2 × 10⁴ cells were seeded into 96-well plates containing 100 μL growth medium in the absence or presence of increasing concentrations of resveratrol and quercetin. After 24-, 48- and 72 hours incubation periods for resveratrol and 72 hours for quercetin, the cells were treated with 20 μL of MTT reagent for 4 hours. Then, the plates were read under 570-nm wavelengths by ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland). Finally, IC50 values of resveratrol and quercetin were calculated from the cell proliferation plots.22

Measurements of caspase-3 enzyme activity

Changes in caspase-3 enzyme activity of the cells treated with increasing concentrations of resveratrol and quercetin for 48 hours were examined by caspase-3 colorimetric assay kit (BioVision Research Products, CA, USA). In short, the cells (1 × 10⁶ cells/2 mL/well) were collected by centrifugation at 1000 rpm for 10 minutes. 50 μL of chilled cell lysis buffer was added onto pellets. The obtained cell lysates were incubated on ice for 10 minutes and centrifuged at 10 000 g for 1 minutes. Supernatants were transferred to new Eppendorf tubes and the reaction mixture containing 50 μL of 2X reaction buffer (containing 10 mM DTT), 50 μL of sample and 5 μL of DEVD-pNA substrate was prepared in 96 well plate and incubated for 2 hours at 37°C in a CO₂ incubator. Then, the plate was read under 405 nm wavelengths by ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland). The absorbance values are normalized to protein concentrations determined by Bradford assay.

Evaluation of apoptosis

For detection of apoptosis, Annexin V-FITC apoptosis detection kit (BioVision Research Products, CA, USA) was used with minor modifications and percentage apoptosis was measured by flow cytometry analysis. 232B4 cells (1 × 10⁶/2 mL/well) were exposed to different concentrations of resveratrol and quercetin for 48 hours. After treatment, cells were collected, washed twice with PBS, and resuspended in 500 μL binding buffer. Later, 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) were added to the suspension and the cells were then incubated in the dark for 15 minutes at room temperature. Samples were then analyzed by flow cytometer. This method is
based on the detection of phosphatidylserine (PS) which is translocated from the inner face of the plasma membrane to the cell surface soon after the induction of apoptosis. PS can be easily detected by staining with a fluorescent conjugate of Annexin V.

**Cell cycle analysis**

The effects of different concentrations of resveratrol and quercetin on cell cycle profiles of 232B4 cells at 48 hours were detected in the presence of DNase-free RNase and propidium iodine (PI) by flow cytometry. For cell cycle analysis, 232B4 cells (1 × 10⁶/2 mL/well) were collected by centrifugation at 1200 rpm for 5 minutes and supernatants were removed. Then, pellets was dissolved with 1 mL of cold PBS. After resuspension of pellets, 4 mL of cold absolute ethanol was added to the pellets with gentle vortexing. The cell suspension was kept at −20°C at least for 1 day. At the day of staining, the samples were centrifuged at 1200 rpm for 10 minutes at 4°C. Supernatants were removed and pellets were resuspended in 5 mL cold PBS. Samples were centrifuged at 1200 rpm for 10 minutes. Then, pellets were dissolved in 1 mL of PBS-triton (100X). 100 μL RNAse-A (200 μg/mL) was added onto the solution and incubated at 37°C for 30 minutes. Finally, the cells were stained with 100 μL PI (1 mg/mL) and incubated at room temperature for 10 minutes. Then, cell cycle profiles of the cells were analyzed by flow cytometry.

**Results**

Resveratrol and Quercetin decreased proliferation of human 232B4 CLL cells in a dose dependent-manner. In order to determine antiproliferative effects of resveratrol and quercetin on 232B4 CLL cells, MTT cell proliferation assay was conducted after 72 hours incubation period of CLL cells with increasing concentrations of Resveratrol and Quercetin. The results displayed that resveratrol and quercetin caused dose-dependent decreases in 232B4 proliferation as compared to untreated control samples. IC50 values of resveratrol (Fig. 1A) and quercetin (Fig. 1B) were found to be 27- and 24 μM, respectively.

Resveratrol and Quercetin induce apoptosis in 232B4 cells. Changes in caspase-3 enzyme activity, loss of MMP and Annexin-V-FITC staining to determine apoptotic cell populations were examined in order to demonstrate apoptotic effects of resveratrol and quercetin on 232B4 CLL cells. As shown in Fig. 2A, there were 2-, 16-, 38-, 49- and 107% increases in caspase-3 activity in response to 0, 5-, 1-, 10-, 50 and 100 μM resveratrol, respectively. 0, 1-, 5, 10 and 50 μM quercetin increased caspase-3 activity 3-, 12-, 38 and 47%, respectively.

**Figure 1**  Cytotoxic effects of resveratrol (A) and quercetin (B) on human 232B4 CLL cells. The IC50 values of resveratrol and quercetin were calculated from cell proliferation plots. The results are the means of three independent experiments. The error bars represent the standard deviations.

**Figure 2**  Changes in caspase-3 enzyme activity in response to resveratrol (A) and quercetin (B). The results are the means of three independent experiments. The error bars represent the standard deviations.
Resveratrol and quercetin induced apoptosis was further confirmed by Annexin V-FITC/PI double staining. Annexin V-positive/PI negative cells were considered early apoptotic, Annexin V-positive and PI positive cells were late apoptotic. After 48 hours of treatment of 232B4 cells with stepwise increasing concentrations of resveratrol (Figs. 3A and 3B) and quercetin (Figs. 4A and 4B), total apoptotic cell population (late apoptotic plus early apoptotic) increased in a dose-dependent manner. There were 2-, 8-, 133-, 350- and 382% increases in apoptotic cells in response to 0.5-, 1-, 10-, 50- and 100 μM resveratrol, respectively, as compared to untreated 232B4 cells (Figs. 3A and 3B). Quercetin increased apoptotic cell population 56-, 133-, 160-, 297- and 331% in response to 0.1-, 1-, 10-, 50- and 100 μM, respectively (Figs. 4A and 4B).

Resveratrol and Quercetin inhibits cell cycle progression. Cell cycle analysis using flow cytometry was carried out to determine if resveratrol and quercetin inhibits cell proliferation. The results revealed that resveratrol application increased percentage of cell population in G0/G1 phase, which was accompanied by a corresponding reduction in the percentage of cells in S phase in a dose dependent manner (Fig. 5). Treatment with quercetin increased the cell population at G0/G1 and decreased the percentage of cells in G2/M phases at the same time as compared to untreated control (Fig. 6).

**Discussion**

In present study, we demonstrated that resveratrol and quercetin inhibits cell proliferation and cell cycle progression and induces apoptosis in CLL cells. IC50 values of resveratrol and quercetin was 27- and 24 μM, respectively. Cytotoxic effects of resveratrol on different types of cells have been reported previously. The IC50 for resveratrol was 45 μM on lung cancer cells and this concentration is effective on many tumor cell types. Resveratrol may possess potential therapeutic activity against lung cancer cells, without significantly affecting the viability of normal cells at its therapeutic concentrations.25 Resveratrol suppressed the colony-forming growth of OCI/AML and OCIM2 cell lines in a dose-dependent fashion at concentrations ranging from 10 to 75 μM. In most studies, resveratrol was used at concentrations ranging from 5 to 100 μM.19 It was reported that quercetin, at a concentration of 20 mM, exhibits an antiproliferative effect on U937 cells.19 Quercetin inhibited cell proliferation in a dose-dependent manner without affecting cell viability. Quercetin-induced inhibition of cell proliferation has been reported for various cancer cells.24

Caspase-3, a key factor in apoptosis execution, is the active form of procaspase-3 whose activation downstream in the apoptotic cascade is essential for leukemia cell apoptosis.26 We used an approach similar to that in a previous study in which we tested the effect of resveratrol on caspase-3 activity in CLL cells. Resveratrol activated caspase-3 in CLL cells. We previously demonstrated activation of caspase-3 enzyme activity in response to resveratrol in imatinib-sensitive and -resistant chronic myeloid leukemia and acute promyelocytic leukemia cells.27 Estrov et al. applied resveratrol and two caspase inhibitors on OCIM2 cells and reported that both caspase inhibitors partially reversed the antiproliferative effect of resveratrol. To further determine whether resveratrol activates the caspase pathway, they incubated OCIM2 cells in the absence or presence of resveratrol. Incubation of OCIM2 cells with resveratrol reduced the levels of procaspase-3, and increased the levels of the biologically active caspase-3.28 Our results were in agreement with the previous studies.

Resveratrol, through different regulatory mechanisms, has been shown to induce apoptosis in tumor cells.29-32 The mechanisms of resveratrol-induced suppression of cellular proliferation have been reported to involve the induction of apoptosis through Fas/CD95, mitochondrial and p53 mediated pathways,29-32 upregulation of CHOP gene expression.33 In addition, resveratrol suppressed NF-kB and protein kinase-C activity, and modulated the angiogenesis and expression of various growth factors as well as NO/NOS.34-38 Our group previously reported that resveratrol triggers apoptosis through up-regulating longevity assurance (LASS) genes that generate apoptotic ceramides and by down-regulating sphingosine kinase-1 and glucosylceramide synthase genes that convert apoptotic ceramides to antiapoptotic sphingosine-1-phosphate and glucosylceramide lipids, respectively, in acute myeloid leukemia and CML cells.12,13 Lee et al. observed that quercetin inhibits cell growth and induces apoptosis in colon cancer cells, and that this may be mediated by its ability to down-regulate ErbB2/ErbB3 signaling and Akt pathway.13 Quercetin-induced apoptosis in SW-872 human liposarcoma cells was associated with the loss of mitochondrial membrane potential. The apoptosis in SW-872 human liposarcoma cells induced by quercetin was mediated through the activation of caspase-3, Bax, and Bak and then cleavage of PARP and downregulation of Bcl-2.15 Kim et al. showed that antiproliferative effect of quercetin is mediated via inhibition of PI-3 kinase in cancer cells.24

Our results revealed that resveratrol treatment induced a dose-dependent increase in the percentage of cells in G0/G1 phase, which was accompanied by a corresponding reduction in the percentage of cells in S phase. A number of studies reported that variety of different human cancer cell lines, treated with resveratrol at micromolar concentrations, arrested the cell cycle progression in S phase39 or less frequently
in the G2/M phase. Resveratrol has been shown to modulate the major cell cycle mediators at micromolar concentrations, arresting cancer cells at the G1/S phase of the different cancer cell. After exposure to 50 μM resveratrol, 64.6% of the AML cells were arrested at the S phase, whereas only 10.9% were at the G2/M phase and 13.6% at the G0/G1 phase of the cell cycle. Parka et al. observed that resveratrol arrested cell cycle progression in the S through G2/M phase in human histiocytic lymphoma U-937 cells. Della Ragione et al. reported that lower concentrations of resveratrol is a differentiation-inducer, causing arrest in the S-phase without subsequent apoptosis in HL60 leukemia cells. Treatment with quercetin increased the cell population at G0/G1 and decreased the percentage of cells in G2/M phases at the same time as compared to control. On the contrary, in previous studies, it was shown that U-937 cells treated with quercetin remarkably accumulated in the G2/M phase of the cell cycle in a time dependent manner up to approximately 80% by 24 hours and U-937 cells were accumulated mainly at G2/M phase when incubated with 50 mM quercetin for 24 hours. Kang and Liang showed that quercetin inhibited the growth of HL-60 leukemia cells in a dose-dependent manner and increase the cell number in G2/M phase.

The results of this study clearly revealed that resveratrol and quercetin induce apoptosis in 232B4 human CLL cells. Furthermore, our results indicated that the growth-inhibitory property of quercetin and resveratrol was mainly due to the induction of apoptosis as evidenced by collapse of mitochondrial membrane potential and activation of caspase-3. These results also suggest that quercetin and resveratrol may be useful for the treatment of human CLL.
Conflicts of interest statement
We, the authors of the manuscript, do not have any conflicts of interest.

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References


