Quercetin elicits proapoptotic effect through downregulation of antiapoptotic and upregulation of proapoptotic genes in human myeloid leukemia KG-1 cells

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily, triggering apoptosis in malignant cell without affecting healthy cells. However, varied types of the cancerous cells (e.g., leukemia) show resistance to TRAIL-induced apoptosis (TIA). Herein, we evaluated the effect of quercetin (QUE) in combination with TRAIL to defeat the resistance of the human acute myeloid leukemia KG-1 cell lines against TIA. Briefly, we treated the KG-1 cells with TRAIL (50 and 250 ng/ml) and QUE (100 μ M) alone and in combination together. Then, the expression levels of the pro-apoptotic proteins, including caspase 3, 8, and 9 along with the anti-apoptotic proteins such as survivin and Mcl-1 expressions levels were evaluated by real-time PCR in KG-1 cells 12, 14 and 48 hours upon exposure with TRAIL and QUE (100 μ M). Based on results, the combination of TRAIL and QUE augmented the expression levels of the caspase 3, 8, and 9 compared with the cells treated with TRAIL and QUE alone. Conversely, expression rates of the survivin and Mcl-1 were strongly diminished in cells treated with TRAIL plus QUE in comparison to KG-1 cells that treated with TRAIL and QUE alone. Considering finding, QUE can not only improve caspase 3, 8, and 9 when used plus TRAIL, it also made a positive effect on their activation due to the inhibitory impacts on anti-apoptotic protein Mcl-1 and survivin expressions in KG-1 cells, possibly leading to the desired therapeutic outcome in leukemic cells.

Keywords: acute myeloid leukemia, apoptosis, quercetin, TNF-related apoptosis-inducing ligand (TRAIL), KG-1 cell line

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INTRODUCTION

Cancer is the abnormal growth of cells in the body and the second leading cause of death in the world. Leukemia is a group of hematologic cancers which initiates the disease in hematopoietic system^{1, 2)}. Despite the high mortality rate of leukemia, no cure has been yet

found that can effectively overcome the diseases³⁾. The primary goal of cancer treatment researches during the past decades has been to develop the strategies to eliminate the cancer cells without affecting normal cells⁴⁾. The selective induction of type I programmed cell death or apoptosis in the malignant cells can be considered as one of the strategies which successfully helps us to eliminate the cancer cells. The apoptosis program can be triggered by either of the intrinsic or extrinsic pathways⁵⁾. The ligation of members of the tumor necrosis factor (TNF) superfamily to its receptor on the cell surface is enough to activate the extrinsic cell death pathway^{6, 7)}. The TNF-related apoptosis-inducing ligand (TRAIL), as a member of the TNF superfamily, has been reported

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that can induce the apoptosis process without affecting the healthy cells in a variety of cancer cell lines⁸⁾. Despite the destructive effects of TRAIL on cancer cells, several studies have revealed that some cancers can acquire resistance to TRAIL-induced apoptosis (TIA)^{9, 10)}. Hence, there are still a lot of barriers to use TRAIL as an anticancer agent. In this regard, more recent studies have confirmed that the combine treatment of TRAIL with the sensitizer agents, such as chemotherapy agents, natural compounds or radiation can be considered as a solution to overcome this problem¹¹⁾. Besides, there is a large volume of published studies describing the pharmacological effects of flavonoids such as the antioxidant activities and the selective antiproliferative roles in cancer cells. Also, several recent findings have shown that quercetin (QUE) is a dietary flavonoid which can trigger the cell death, mainly by initiating the apoptosis process in leukemia cells without affecting the normal cells^{12, 13)}. Based on investigation, QUE could suppress human HL-60 promyelocytic leukemia cell proliferation and promote their apoptosis percentages by targeting protein kinase C (PKC) and tyrosine protein kinase (TPK) pathways¹⁴⁾. Another study indicated that QUE ameliorated mitochondrial function by stimulating the proteasomal degradation of Mcl-1 (myeloid cell leukemia 1), a pro-survival member of the Bcl-2 family and by suppression of the expression of survivin, a member of the inhibitor of apoptosis (IAP) family, in non-Hodgkin's lymphoma¹⁵⁾. Furthermore, it has been evidenced that QUE may be valuable therapeutic approach to treat human leukemia U937 cells by triggering apoptosis in leukemia versus normal hematopoietic cells by a procedure, encompassing Mcl-1 down-regulation, supporting Bax activation and mitochondrial translocation, and thereby culminating apoptosis¹⁶⁾. Besides, reports revealed that QUE down-regulated Mcl-1 by the suppression of PI3K/Akt signaling pathway, promoting Mcl-1 instability in leukemia cells, which in turn, led to the apoptosis¹⁷⁾.

In the present study, we have co-treated the human myeloid leukemia KG-1 cell lines with TRAIL and QUE. Then, we investigated whether this co-treatment can change the messenger RNA (mRNA) expression levels of Caspase-3, 8, and 9 and the apoptosis inhibitors including survivin and Mcl-1 or not.

MATERIALS AND METHODS

Cell culture

Human myeloid leukemia KG-1 cells were purchased from General Cell Collection Bank of Iran (Pasteur Institute, Tehran, Iran). The KG-1 cell line maintained in exponential growth in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, Gillingham, UK), 50 µg/ml of streptomycin (Sigma-Aldrich), and 50 units/ml of penicillin

(Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents

A 100 mM stock solution of QUE (>99% pure; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO), stored at -20°C, and then diluted as needed in RPMI-1640 to its final concentration in each culture dish. Also, a 0.5 mg/ml stock solution of the recombinant human Apo2L/TRAIL (Merck, Darmstadt, Germany), was prepared in water. RPMI-1640 medium, FBS, penicillin, streptomycin, and phosphate buffered saline were purchased from Gibco (Carlsbad, USA). DMSO was obtained from Sigma-Aldrich.

RNA extraction and Quantitative real-time polymerase chain reaction

The KG-1 cells treated with TRAIL (50 and 250 ng/ml) and QUE (100 µM) alone and in combination at the 12, 24, and 48 hours. Then, the cells harvested for RNA extraction using Trizol Reagent (Invitrogen, Milan, Italy) following the manufacturer's instructions. The quality and concentration of the RNA samples was assessed by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The recommended RNA quality parameters for Real-time PCR analysis were as follows: UV spectroscopy A260/ A280 ratio of 1.8–2.0. 500 nanograms of the extracted RNA were reverse-transcribed into cDNA using, PrimeScript™ reagent Kit (Takara Bio, Kusatsu, Japan). Real-time PCR analysis was done using the using the RealQ Plus 2x Master Mix Green (Ampliqon, Herlev, Denmark) following the manufacturer's protocol. Quantification and the analysis of melting curves was performed using the real-time PCR system (Rotor-Gene 6000; Corbett Life Science, Sydney, Australia). Also, we used the primer-blast software on NCBI website (<http://www.ncbi.nlm.nih.gov>) to Basic Local Alignment Search Tool (BLAST) our all primer sequences before the experiment. Beta-actin (ACTB) was used as an internal control. The primers used in this study and target sequences data listed in Table 1.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 8.3.0 for Windows (GraphPad Software Inc, San Diego, USA). Gene expression data was analyzed for any statistically significant using a two-way analysis of variance with the post hoc Dunnett and Tukey multiple comparison tests. Data were presented as the mean ± standard error of the mean (SEM) and were representative for three separate experiments. P values <0.05 were considered to be statistically significant.

Table 1. Primer Sequences, All of the sequences of the primers are presented in 5' to 3' orientation.

Gene	Forward Primers (5' - 3')	Reverse Primers (5' - 3')
Caspase 3	TGTTTGTGTGCTTCTGAGCC	CACGCCATGTCATCATCAAC
Caspase 8	GATCAAGCCCCACGATGAC	CCTGTCCATCAGTGCCATAG
Caspase 9	CATTTCATGGTGGAGGTGAAG	GGGAAGTGCAGGTGGCTG
Survivin	TTCTCAAGGACCACGCATC	GCCAAGTCTGGCTCGTTCTC
Mcl-1	AGAAAGCTGCATCGAACCAT	CCAGCTCCTACTCCAGCAAC
ACTB	TGAAGATCAAGATCATTGCTCCC	AGTCATAGTCCGCCTAGAAGC

RESULTS

QUE increased the expression levels of Caspase 3 gene in the human myeloid leukemia KG-1 cells (at transcriptional level)

To evaluate the QUE effect on caspase 3 expression in KG-1 cells, which constitute an in vitro model of human acute myeloid leukemia, we employed the quantitative real-time polymerase chain reaction analysis to compare the QUE treated and untreated cells. Based on the results, the caspase 3 expression did not change significantly in cells treated with TRAIL alone during 12, 14 and 48 hours of treatment compared with the control group ($P < 0.05$); however, its expression levels were significantly augmented in KG-1 cells treated with QUE (100 μ M) alone and in combination with TRAIL (50 and 250 nM) during 12, 14 and 48 hours of treatment. Moreover, the

expression levels of caspase 3 were more elevated in cells treated with QUE in combination with TRAIL during 12 and 24 hours than 48 hours of exposure. The results are shown in Fig. 1.

QUE increased the expression levels of caspase 8, and 9 genes in the human myeloid leukemia KG-1 cells (at transcriptional level)

In this regard, to investigate the effect of QUE on caspase 8, and 9 as the initiator caspases in apoptosis, we evaluated their mRNA expression levels after treating the KG-1 cells with TRAIL and QUE during the indicated times. Although the caspase 8, and 9 expression did not modify significantly in cells treated with TRAIL alone during 12, 14 and 48 hours of treatment, their expression levels were significantly promoted in KG-1 cells treated with QUE (100 μ M) alone and in combination with TRAIL (50 and 250 nM) during 12, 14 and 48 hours of

Fig. 1. Real-Time PCR data for caspase 3 expression in KG-1 cells treated with TRAIL (50 and 250 nM) and QUE (100 μ M) alone and in combination within 12, 14 and 48 hours of treatment. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM. ACTB was selected as internal control. One-Way ANOVA was used to evaluate the observed statistical differences. P values < 0.05 were considered statistically significant.

Fig. 2. Real-Time PCR data for caspase 8 (A) and caspase 9 (B) expression in KG-1 cells treated with TRAIL (50 and 250 nM) and QUE (100 μ M) alone and in combination within 12, 14 and 48 hours of treatment. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM. ACTB was selected as internal control. One-Way ANOVA was used to evaluate the observed statistical differences. P values <0.05 were considered statistically significant.

Fig. 3. Real-Time PCR data for Mcl-1 (A) and survivin (B) expression in KG-1 cells treated with TRAIL (50 and 250 nM) and QUE (100 μ M) alone and in combination within 12, 14 and 48 hours of treatment. Data are representative of three independent experiments and values are expressed in mean $\pm$ SEM. ACTB was selected as internal control. One-Way ANOVA was used to evaluate the observed statistical differences. P values <0.05 were considered statistically significant.

treatment compared with the control group ($P < 0.05$). Furthermore, the expression levels of the caspase 8 and 9 were higher in KG-1 cells treated with QUE in combination with TRAIL within 48 hours than the 12 and 24 hours of treatment. The real-time PCR results are shown in Fig. 2.

QUE decreased the expression of apoptosis inhibitor genes in the acute myeloid KG-1 cells (at transcriptional level)

Finally, in this part, we attempted to investigate the

effect of quercetin on the expression levels of Mcl-1 and survivin at transcriptional levels in KG-1 cells treated with TRAIL (50 and 250 nM) and QUE (100 μ M) alone within 12, 14 and 48 hours of exposure. Many researchers have argued that apoptosis inhibitors can act as an important factor in developing cancer in the abnormal cells. As shown in Fig. 3A, the QUE treatment of KG-1 cells has been able to significantly reduce the mRNA expression levels of Mcl-1 either in QUE alone or in combination with TRAIL-treated cells during the indicated times compared with the control group ($P < 0.05$). The

survivin gene expression changes that has been plotted in Fig. 3B clearly shows that these changes are roughly similar to the Mcl-1 gene at the transcriptional level compared with control group ($P < 0.05$). Considering analysis, inhibitory effects of the QUE on Mcl-1 and survivin expression were stronger in 48 hours than 12 and 24 hours of exposure.

DISCUSSION

TRAIL, as a biological cytokine, is attracting attention as a potential therapeutic agent that selectively can trigger apoptosis in cancer cells but not in normal cells^{18, 19}. But the therapeutic usage of this cytokine is limited due to resistance of some cancer cells to the TRAIL induced apoptosis (TIA)^{20, 21}. Hence, introducing new sensitizer agents can sensitize resistant cancer cells to TIA. Recent evidences suggest that QUE as a plant flavonol can be used as a sensitizing agent alongside with TRAIL in some cancer cells^{22, 23}. In this context, Kim and his coworkers found that QUE promoted TIA by suppression of survivin expression, through ERK-MSK1-mediated deacetylation of H3 in human prostate cancer cell lines DU-145 and PC-3. They suggested that up regulation of caspase-8, -9, and -3 along with down regulation of survivin could remarkably stimulate apoptosis in treated cells with QUE plus TRAIL²⁴. Similarly, another study conducted by Psahoulia et al. showed that QUE plus TRAIL triggered the apoptosis intrinsic pathway, as presented by Bid cleavage and the cytochrome c release to the cytosol, accompanied by redistribution of DR4 and DR5 into lipid rafts in colorectal cancer (CRC)²⁵. Moreover, QUE plus TRAIL augmented apoptosis in hepatocellular carcinoma (HCC) cells upon DR5 up regulation in a transcription factor Sp1-dependent manner in concomitant with down regulation c-FLIP, an inhibitor of caspase-8, by proteasome-exerted degradation²⁶. As well, suppression of survivin expression and activation in association with DR5 up regulation were considered as corresponding mechanisms participated TRAIL-mediated apoptosis in non-small cell lung cancer (NSCLC) cells upon exposure with QUE, according to Chen et al. reports²⁷. However, the mechanism of action of QUE in sensitizing leukemia cells (e.g., KG-1) to the TIA is not well understood. In our recent study we demonstrated that QUE alone or alongside with the use of TRAIL could augment the TIA in the resistant acute myeloid KG-1 cells to TIA²⁸. Analysis revealed that quercetin represses the expression of NF- κ B (p65 subunit) gene in KG-1 cells²². This finding is consistent with findings of other studies that are emphasizing on the possible role of the inhibition of NF- κ B for the caspase-3 upregulation. The NF- κ B prevents apoptosis through increasing the expression of several Bcl-2 family members genes that these targets prevent apoptosis²⁹. It can be concluded

that among the factors that have been able to increase the rate of apoptosis in KG-1 cells are NF- κ B targets that affect the release of cytochrome c from mitochondria. Additionally, the inhibitory effect of quercetin on NF- κ B expression, leading to the increased expression of caspases including caspases 3, 8 and 9, may be one of the main mechanisms in sensitizing KG-1 cells to TIA. The aim of the present work is to investigate the further anti-cancer effects of QUE on KG-1 cells. Accordingly, firstly, we evaluated expression levels of anti-apoptotic proteins Mcl-1 and survivin in the KG-1 cells upon exposure with TRAIL and QUE alone and in combination. It has been already been found that overexpression of anti-apoptotic proteins, in particular Mcl-1 and survivin, is one of the main reason of cancer cells resistance to TIA^{30, 31}. Considering molecular analysis, we found that QUE stimulated an inhibitory effect on Mcl-1 and surviving expression levels when used along with TRAIL. There exist a consistency between these results and other findings in varied types of the cancer cells (e.g., leukemia). For example, Jacquemin et al. found that QUE induced TIA in resistant transformed follicular lymphoma B-cell lines through the triggering the proteasomal degradation of Mcl-1 accompanied by suppressing survivin expression, irrespective of p53¹⁵.

Secondly, examination of QUE and TRAIL effects on mRNA levels of the pro-apoptotic factors, such as caspase 3, 8, and 9, delivered the proof of concept that QUE can triggered TIA in KG-1 cells by amelioration of the activator and effector caspase expression. These findings are consistent with the results of previous studies exploring the effect of QUE on TIA in other tumor cells³².

In sum, studies on the anti-leukemic potential of flavonoids are described a stimulating topics of the context of the leukemia therapy because of their lower unwanted effect and notable safety. In the present study, we found that QUE can robustly promote aspase-3, -8 and -9 expression in leukemia KG-1 cells; on the other hand, it stimulated a reduction in anti-apoptotic protein Mcl-1 and survivin expression in mRNA levels, which in turn can lead to the apoptosis induction in treated cells. Accordingly, we guess that QUE can be a rational drug-candidate to progress the novel anti-leukemia therapeutics toward a spectrum of leukemia with minimum side-effects and suitable rates of safety along with acceptable efficacy.

Declaration of competing interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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