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Evaluation of BAX and BCL-2 Gene Expression Levels and Apoptosis in Resveratrol Affected Human Leukemic Cell Line: CCRF-CEM

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Abstract

Objectives: Acute lymphoblastic leukemia (ALL) is considered one of the common types of cancers in childhood with an incidence of up to 25%. In addition, drug resistance is a phenomenon which reduces the chances of overcoming cancer. Further, a phytoalexin combination called resveratrol can sensitize the leukemic cells to apoptotic cell death. Due to the importance of the above-mentioned issues, the present study aimed to evaluate the effect of resveratrol on BAX and BCL-2 expression levels and apoptosis induction.

Materials and Methods: CCRF-CEM cultured cells were treated by resveratrol at doses of 15, 50, and 100 µM based on previous studies. Furthermore, RT Polymerase chain reaction (PCR) was conducted to assess the BAX and BCL-2 gene expression. Moreover, the amount of apoptosis induction was analyzed by annexin V staining method.

Results: Based on the results, time and concentration were found to play a critical role in resveratrol-induced apoptosis. Additionally, BAX upregulation and BCL-2 downregulation excreted by resveratrol in CCRF-CEM cells resulted in predisposing these cells to apoptosis.

Conclusions: In general, it was revealed that resveratrol could have a chemo-preventive activity by modifying the expression of BAX and BCL-2 genes. Finally, resveratrol was found to be a supplement drug in anti-leukemic therapy.

Keywords: Leukemia, Resveratrol, BCL-2 associated X, BCL-2 Gene, Apoptosis

Introduction

Acute lymphoblastic leukemia (ALL) is a hematologic neoplasm of leukocytes, which comes mainly from the bone marrow. Totally, ALL accounts for 25% of all types of cancer incidence and is one of the common cancers during childhood (1,2). T-acute lymphoblastic leukemia (T-ALL) is a malignant and progressive neoplasm which originates from precursors of the T cell. Nowadays, about 15 and 25% of ALL incidence in children and adults belong to T-ALL (3).

Recurrence phenomenon in T-ALL cases is approximately 30% and the response to treatment is still poor (3-5). Thus, applying new therapies to these patients is of an urgent need (3). Now, many studies have emphasized that apoptosis susceptibility of cancerous cells is very important in identifying the effectiveness of chemotherapeutic agents, therefore the presence of apoptosis disorders which leads to an inappropriate response to treatment and outcomes is poor in patients with T-ALL (5,6). Based on the above-mentioned data, the CCRF-CEM cell line was selected as a chemo-resistant cancerous cell in the present study. Natural products are invaluable resources compared to pharmaceuticals. Nowadays, more than half of the existing drugs are developed from natural compounds or their derivatives. Natural compounds include nearly 60% of all the compounds used in cancer treatment (1,7). Resveratrol (trans-3, 4', 5-trihydroxystilbene) is considered a phytoalexin which is found in several herbal compounds, especially in the red grapes and it has been introduced as an anti-cancer agent since 1997 (3,8). In addition, resveratrol can be obtained from seventy different species of plants, including grapes, blueberries, peanuts (9,10). A large number of studies confirmed various properties of resveratrol including preventing the proliferation, inhibiting the angiogenesis, preventing the emission

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factors, as well as tumor progression and metastasis. Anti-cancer properties of resveratrol in different cancers revealed that its mechanism of action depends on the cell type involved in cancer (3,11). Further, the potential benefits of it prove that resveratrol can inhibit cell growth and induce apoptosis in several cell lines, such as prostate cancer, leukemia, colon cancer, and breast cancer (9,12-14). Apoptosis is an essential mechanism of cell death, which is considered as the main goal in cancer treatment.

The molecular pathways which are affected by resveratrol are not completely understood. However, some previous studies have highlighted several effects of resveratrol, for example, in intervening in MAPK and PKC pathways, inhibiting ribonucleotide reductase, DNA synthesis, as well as cyclooxygenase activity, changing the miRNA expression, upregulating the expression of receptors TRAIL 1 and TRAIL 2, decreasing the expression of NF-kappa B, IKK 1, cyclin D, and increasing the expression of P21 (1,7,9,15).

BCL-2 family members are divided into pro-apoptotic and anti-apoptotic proteins which have a critical role in keeping the balance of the apoptosis process (16). Disruption in the ratio of these two proteins leads to a defect in apoptosis in the affected cells. Furthermore, overexpression of BCL-2 and down-regulation of BAX expression indicate that these genes can be involved in ALL pathogenesis (17). The main mechanism of resveratrol anticancer properties in different types of cancer cells is inducing the apoptosis and alteration the gene expression related to anti-apoptotic proteins (18-21). Based on the above-mentioned explanations, the current study sought to investigate the effect of resveratrol on BAX and BCL-2 gene expression levels in order to elucidate one of the pathways which resveratrol may alter to induce the apoptosis. As it is known, the effect of Bcl-2 overexpression is extremely important forming chemo-resistant leukemia.

Materials and Methods

Cell Culture

The T-ALL cells (CCRF-CEM) were obtained from the Pasteur Institute (Tehran, Iran). Based on the methods used in previous studies (22,23), the cell line was cultured at carbon dioxide 5% and 37°C. Moreover, the medium of RPMI 1640 contained 10% fetal bovine serum (FBS, GIBCO, USA) and 100 U/mL antibiotic (penicillin-streptomycin, GIBCO, USA).

Treatment

Resveratrol (purity >99%) was obtained from Sigma Aldrich (Sigma Aldrich, Germany) and dissolved in vehicle control (ethanol). CCRF-CEM cells were then seeded in 6-well plates with RPMI 1640 medium. Additionally, resveratrol was added at 15, 50, and 100 µM doses. Finally, the cells were harvested for RNA extraction 24 and 48 hours later (5,24).

RNA Extraction

TRIzol reagent was used for RNA extraction (Invitrogen, USA) based on manufacturer instructions. In addition, the samples were accepted for the cDNA synthesis based on the A260/A280 ratio which lies between 1.8 and 2.0 (22).

Real-time PCR

After 24 and 48 hours of treatment (5), cDNA was synthesized based on the instruction of RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA). Further, real time polymerase chain reaction (PCR) analysis of BAX and BCL-2 genes was performed using a SYBER Green qRT-PCR kit (Invitrogen, USA) based on the instruction of the manufacturer. Furthermore, β-actin was applied as an internal reference. The primers employed in real-time PCR were as follows:

- BAX forward primer: 5'-GCCCTTTTGCTTCAGGGTTT-3';
- BAX reverse primer: 5'-TCCAATGTCCAGCCCATGAT-3';
- BCL-2 forward primer: 5'-CGGAGGCTGGGATGCCTTTG-3';
- BCL-2 reverse primer: 5'-TTTGGGGCAGGCATGTTGAC-3';
- β- actin forward primer: 5'-GAGACCTTCAACACCCCAGCC-3';
- β- actin reverse primer: 5'-AGACGCAGGATGGCATGGG-3'.

The whole process was repeated three times (5).

Annexin V Staining for Apoptosis Assays

The annexin V staining in CCRF-CEM cells was examined by flow cytometry method using the FACS machine in order to evaluate the apoptosis. Moreover, treatment was conducted using different concentrations (15, 50, and 100 µM) and then the cells were incubated for 24 and 48 hours at 37°C. Ice-cold PBS was used to wash the harvested cells. Next, based on the manufacturer's manual, cells were resuspended with binding buffer containing annexin V after centrifugation. Finally, FACS flow cytometer (BD FACScaliber, USA) was employed to detect and analyze the early apoptosis incident (25).

Statistical Analysis

In the current study, all techniques were conducted in triplicate. Student's t-test was applied to determine the statistical differences between control and test groups. The $P<0.05$ was considered statistically significant.

Results

Altering the Levels of BAX and BCL-2 Gene Expression by Resveratrol

Based on the results, resveratrol altered BAX and BCL-2 expression in CCRF-CEM cell line as a model of T-ALL evaluated by RT PCR technique. Additionally, the BAX

expression level was up-regulated while the BCL-2 expression level was decreased in resveratrol-treated cells after different incubation times. Expression changes in the above-mentioned genes exerted by resveratrol in a time and dose-dependent manner (Figure 1). However, the first time of treatment was excluded due to its low effect on the expression of BAX and BCL-2 in the Annexin V staining for evaluating apoptosis induction. Eventually, it was found that resveratrol can alter the BAX/ BCL-2 ratio and accordingly, predispose the chemo-resistant cells to apoptosis.

The Increase of Apoptotic Cells by Resveratrol Treatment

Annexin V staining method was used to detect apoptotic cells which were induced by different doses of resveratrol. As illustrated in Figures 2 and 3, apoptosis is induced by different concentration of resveratrol (15, 50 and 100 µM). Taken together, the results indicated that apoptosis induction significantly increased after 48 hours compared to 24 hours. Therefore, time is regarded as an important item in inducing apoptosis by the resveratrol (Figures 2 and 3).

Discussion

ALL is regarded as one of the common malignancy in childhood and chemotherapy is one of the widespread therapies for its treatment. However, resistance to chemotherapy may decrease the effect of treatment (1). Overexpression of Bcl-2 is one of the pathways through which the cancerous cells resist chemotherapy (26). As a result, in the current study, the effects of resveratrol on the expression level of BAX and BCL-2 genes were investigated. Sensitizing the cancerous cells to routine cancer drugs such as glucocorticoids is believed to be one of the strategies for overcoming drug resistance (27). In addition, apoptosis induction is the main

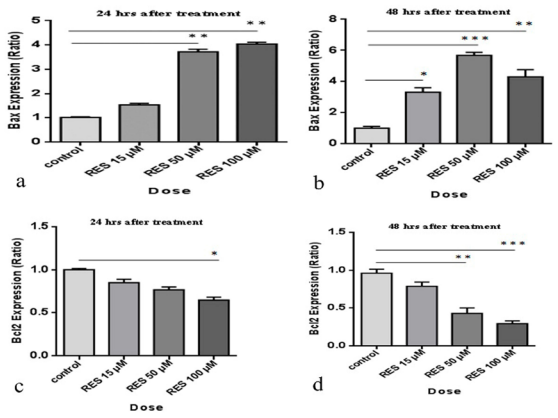

Figure 1. The altered gene expression of BAX and BCL-2 which were induced by resveratrol and analyzed by RT-PCR. Internal control for gene variation was β-actin. (a) The BAX expression level after 24 hours; (b) The BAX expression level after 48 hours; (c) The BCL-2 expression level after 24 hours; (d) The BCL-2 expression level after 48 hours. *Note*. ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

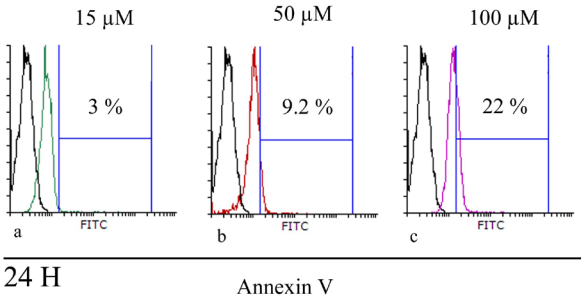

Figure 2. Annexin V Staining Results by Means of Flow Cytometry Method After 24 Hours of Incubation: (a) 15 µM of resveratrol; (b) 50 µM of resveratrol; (c) 100 µM of resveratrol. *Note*. Control: Ethanol (vehicle control).

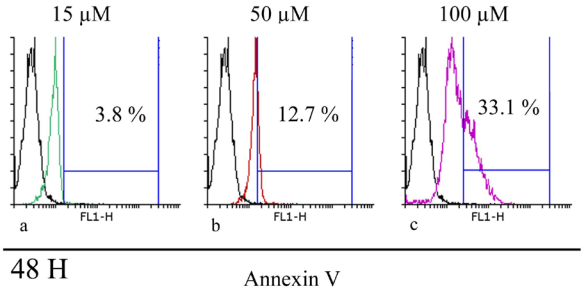

Figure 3. Annexin V Staining Results by Means Flow Cytometry Method, After 48 Hours Incubation: (a) 15 µM of Resveratrol, (b) 50 µM of Resveratrol, (c) 100 µM of Resveratrol. Control: Ethanol (vehicle control).

goal of anti-cancer therapy in various neoplasms and chemoprevention is considered an application to reduce the dose of chemotherapy agents (5,28,29). Based on the reports of several trials, a wide variety of natural materials or foodstuffs can inhibit cancer (30). However, different studies mainly focused on a natural phytoalexin called resveratrol which includes a whole range of different biological effects such as antiproliferative and anti-inflammatory, as well as natural chemo-preventive activity against human cancers (31). Further, resveratrol suppresses cell growth through inducing apoptosis in the transformed cells (32,33). Furthermore, resveratrol is introduced as a new supplemental drug against a wide range of cancers (30). The results of the present study regarding apoptosis is in line with those obtained in our previous study (1) and raises this issue that apoptosis induction by resveratrol in CCRF-CEM cell line is conducted in a time and dose-dependent manner. Moreover, results suggest that resveratrol apoptotic properties may be related to BCL-2 down-regulation and BAX up-regulation. The effect of resveratrol on different cells varies. For example, Brito et al indicated that resveratrol decreased BAX/ BCL-2 ratio through increasing BCL-2 expression in endothelial cells (34). Therefore, the effects of resveratrol on different cell lines are subject to further investigation. Park et al demonstrated that resveratrol cannot effectively induce apoptosis in over-expressed Bcl-2 U937 leukemic

cells. They found that overexpression of Bcl-2 disrupts the release of cytochrome C in this transfected cell line and thus the resveratrol affected U937 cells resist apoptosis (35). The findings of these studies justify the aim of the present research for determining the role of BAX and BCL-2 genes in apoptosis induction in resveratrol affected CCRF-CEM cell lines. Finally, the current study confirmed the anti-cancer effects of resveratrol in treating the relapse model of T-ALL cells (CCRF-CEM). Additionally, it was revealed that resveratrol causes BAX up-regulation, and BCL2 down-regulation in a dose- and time-dependent manner, and therefore can induce apoptosis through the modification of the apoptotic proteins of BCL2 family.

Conclusions

In general, it is believed that resveratrol can be employed as an effective herbal compound for T-ALL treatment. However, further studies should be implemented in order to better understand the effects of resveratrol on different cellular signaling pathways and discover therapeutic strategies in this regard.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

This study was approved by Ethic committee of Maragheh University of Medical Sciences (No. IR.MRGUMS.REC.1386.29).

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