RESEARCH ARTICLE

Time - and Concentration - Dependent Effects of Resveratrol on miR 15a and miR16-1 Expression and Apoptosis in the CCRF-CEM Acute Lymphoblastic Leukemia Cell Line

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Abstract

Background: Chemotherapy is one of the common approaches in treatment of cancers, especially leukemia. However, drug resistance phenomena reduce the likelihood of treatment success. Resveratrol is a herbal compound which through complicated processes makes some selected cells sensitive to treatment and induction of apoptosis. In the present study, the effects of resveratrol on the expression of miR 15a and miR16-1 and apoptosis in the CCRF-CEM cell line were investigated. Materials and Methods: The CCRF-CEM cell line was cultured under standard conditions and changes in miR 15a and miR 16-1 expression were analyzed by real time-PCR technique, with attention to reveratrol dose and time dependence. Also, apoptosis is evaluated by flow cytometry using annexin V and PI. Results: CCRF-CEM cells underwent dose-dependent apoptotic cell death in response to resveratrol. MiR 15a and miR 16-1 expression was up-regulated after 24 and 48 hours resveratrol treatment ($p<0.05$). Conclusions: The results of our study indicate that resveratrol induces apoptosis in a time and dose-dependent manner in CCRF-CEM cells. Also, increased expression level of miR 16-1 and miR 15a by means of resveratrol in CCRF-CEM cells might have a role in apoptosis induction and predisposition. According to our results resveratrol can be regarded as a dietary supplement to improve efficacy of anti-leukemia therapies.

Keywords: ALL - resveratrol - miR 15a - miR 16-1 - apoptosis

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Introduction

Acute lymphoblastic leukemia (ALL) is a rapidly growing aggressive cancer of White Blood Cells (WBC) that mostly originates from bone morrow and is the most prevalent pediatric cancer (Pui et al., 2008; Stanulla et al., 2009). Natural products are a rich source of valuable medicinal agents. More than half of the currently available drugs are natural or related compounds. In the case of cancer therapy, the percentage of natural compound exceeds 60% (Keitaro et al., 2012). Resveratrol (trans-3, 4', 5-trihydroxy stilbene) is produced by several plants in response to stress, injury, UV irradiation and fungal infections. Resveratrol is normally found in many dietary products such as grapes, peanuts, berries and wine (Signorelli et al., 2005). Numerous pharmacological properties have been attributed to resveratrol including antioxidant, anti-inflammatory, cardio-protective, and anti-proliferative activities (Fremont., 2000; Fulda ., 2010; Pallares et al., 2012; Panaro et al., 2012; Lin et al., 2013; Sevinc et al., 2013; Cullberg et al., 2014; Gaung et al., 2014; Lephart et al., 2014). previous studies have been shown that growth inhibition and apoptosis induction properties of Resveratrol in several malignant cells, suggested anti-cancer properties of this compound (Hai et al., 2013; Yang et al., 2013; Yu et al., 2013; Chin et al., 2014; liu et al., 2014; Xin-pin et al., 2015). The molecular mechanisms of Resveratrol are not fully defined, although some reports indicate that it interferes with the MAPK and PKC pathways (Clement., 1998; Yu et al., 2001; Lin et al., 2003; Jei et al., 2012), ribonucleotide reductase and DNA synthesis inhibition (Fontecave et al., 1998), cyclooxygenase activity inhibition (Subbaramaiah et al., 1998), alteration of miRNAs expression (Keitaro et al., 2012), up-regulation of TRAIL receptor 1 and 2 expression, down-regulation of Bcl-2 expression, down-

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regulattion of NF-kappa B and IKK1 expression, decrease the expression of cyclin D and increase the expression of p21 (Sevinc et al., 2013; Ulasli et al., 2013).

The human genome contains several hundred miRNAs. These are noncoding RNAs, typically 20-24 nucleotides in length. MiRNAs regulate the expression of about 30% of human genes by either inhibiting mRNA translation or inducing its degradation (Bartel., 2004; Lewis et al., 2005). MiRNAs are created from primary transcripts, termed pri-miRNAs, via endonuclease cleavage processing (Bartel., 2004). MiRNAs influence a variety of key biological process, including development, differentiation, apoptosis, survival, senescence, metabolism and signal transduction (Ambros., 2004; He et al., 2004; Chivkula et al., 2008; Inui et al., 2010). MiR15a and miR 16-1, are located on chromosome band 13q14 and are down-regulated in the majority of patient with CLL (Calin et al., 2002). Apoptosis is a cell death mechanism that may be prompted by several molecular pathways, among which the intrinsic and extrinsic (also known as "*death receptor pathway*") pathways are the best known (Juan et al., 2012). MiR 15a and miR 16-1 induce apoptosis through the negative regulation of the anti-apoptotic gene BCL2 (Cimmino et al., 2005). In the other study, show that expression of miR 15a and miR 16-1 is regulated by E2F transcription factor and inhibition of both miR 15a and miR 16-1 enhance E2F1-induced G1/S transition. miR 15a also inhibits expression of cyclin E, a Bona fida target of E2F (Matan et al., 2011). So, increased expression of miR 15a and miR 16-1, induce cell cycle arrest. In this study, we want to evaluate the effect of resveratrol on miR 15a, miR 16-1 and apoptosis in CCRF-CEM cell line. Up-regulation of these two miRNAs can be regarded as apoptosis predisposition.

Materials and Methods

Cell culture: The CCRF-CEM cell line was purchased from Pasteur Institute of Iran (Tehran, Iran), and cultured in RPMI 1640 medium (GIBCO, USA) containing 10% FBS (GIBCO, USA) and antibiotic(containing 10,000 U/mL penicillin and 10 mg/mL streptomycin) at 37˚c in a humidified chamber containing 5% CO_2. cells were seeded into 60mm culture dishes (4 x10^5 cells per dish) before Resveratrol treatment.

Treatment: Resveratrol (98% purity, Sigma-Aldrich, Germany) was dissolved in Ethanol and added to CCRF-CEM cultures at concentrations of 15, 50 and 100 μM. Finally cells were harvested after 24 and 48 hours for RNA extraction.

RNA extraction: Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacture's protocol. For the RT PCR studies, both the quality and concentration of the RNA sample was determined using NanoDrop 2000c UV-Vis spectrophotometer (THERMO, USA). The recommended RNA quality parameters for RT PCR analysis were as follows: UV spectroscopy A260/A280 ratio of 1.8-2.0 and A260/A230 ratio greater than 1.8, 18S/28S rRNA ratio of 1.8-2.1.

cDNA synthesis and Real-Time PCR (RT-PCR): Complementary DNA was synthesized from total RNA using a miRNA cDNA synthesis kit (Invitrogen, USA) according to the manufacture's instruction. In brief, firstly poly A tailing of all the miRNAs were synthesized in a total RNA sample and then, cDNA synthesized from the poly A tailed population. Expression levels of miRNA were determined using an EXPRESS SYBR Green miRNA qRT-PCR kit (Invitrogen, USA) according to the manufacture's protocol. PCR reaction was performed using the Ncode Universal reverse primer (Invitrogen) in conjunction with specific forward primer for each miRNA (Takapouzist, Iran). HRNU6 was used as an invariant control. The β-actin housekeeping gene was used to normalize the variation in the cDNA levels. The PCR forward primer sequences were custom- designed based on mature miRNA sequences (http://www.mirbase.org) . The following primers were used respectively; miR 15a forward primer: 5'-GCCTGTAGCAGCACATAATGG-3'; miR 16-1 forward primer: 5'-CCAGTATTAACTGTGCTGC-3'; U6 reverse primer: 5'-AATTTGCGTAACGCTTCACG-3'; U6 forward primer: 5'-TCCGCTTCGGCAGCACA-3'. All reaction were performed in triplicate.

Apoptosis analysis by Annexin V and PI staining: Cells undergoing apoptosis were identified by Annexin V and Propidium Iodide (PI) staining (Exbio, CZ) according to the manufacture's protocol. Phosphatidylserine, which is normally located on the cytoplasmic surface of cell membrane, is exposed on the cell surface upon induction of apoptosis. Annexin V binds to phosphatidylserine and is used to identify the earliest stages of apoptosis. PI, which does not enter vital cells with intact membranes, is used to distinguish between early apoptotic cells (Annexin V-positive) and late apoptotic or necrotic cells (Annexin V-PI double positive). Briefly, 2-5x105 cells were washed once in cold PBS and then washed cells centrifuge were performed again and discarded supernatant. After that, resuspended cell pellet in 1x Annexin V Binding Buffer. Next, added 5 μL of Annexin V-FITC and 5 μL of PI to each 100 μL cell suspension and mixed gently and incubated cells for 15 min at room temperature in a dark

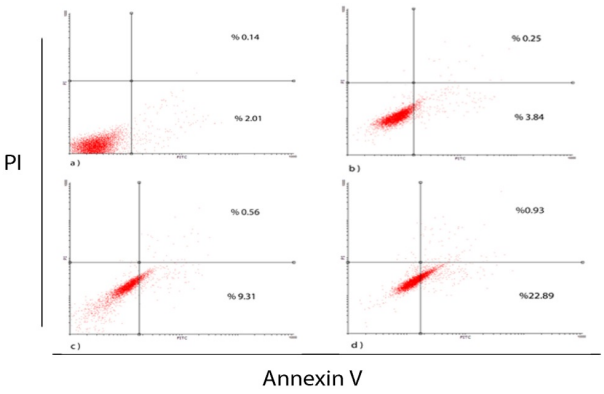

Figure 1. Representative Data of Dual Staining for Annexin V- PI Uptakes from Control and Test Groups by means of FACS, after 24h Incubation. The percentage of Annexin V-positive and PI-negative cells that were in early-stage apoptosis (bottom right) and Annexin V-positive and PI-positive cells that were dead or in end-stage apoptosis (top right), are presented in each quadrant. a) control, b) 15 μM RES, c) 50 μM RES, d) 100 μM RES. control: Ethanol (vehicle control), RES: Resveratrol

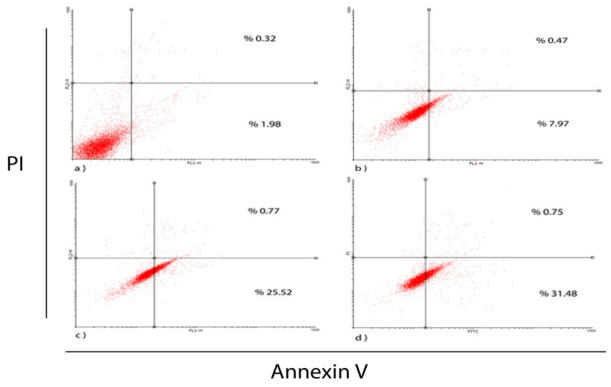

Figure 2. Representative Data of Dual Staining for Annexin V- PI Uptakes from Control and Test Groups by means of FACS, After 48h Incubation. The percentage of Annexin V-positive and PI-negative cells that were in early-stage apoptosis (bottom right) and Annexin V-positive and PI-positive cells that were dead or in end-stage apoptosis (top right), are presented in each quadrant a) control, b) 15 μM RES, c) 50 μM RES, d) 100 μM RES. control: Ethanol (vehicle control), RES: Resveratrol

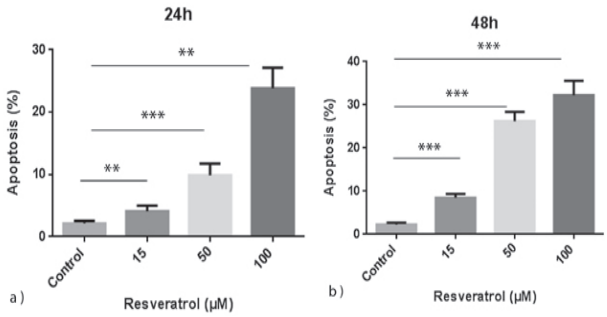

Figure 3. Resveratrol Induced Apoptosis in a dose and Time Dependent Manner. CCRF-CEM cells were treated with Resveratrol (15, 50, and 100 μM) and induced apoptosis in a dose dependent manner after 24h (a) and 48h (b)

place. After the incubation, centrifuged and resuspended pellet in 100 μL of 1x Annexin V Binding Buffer. Finally, the cells analyzed by flow cytometery (BD FACScaliber, USA).

Statistical analysis: The statistical calculations were performed with SPSS 16.0 software (SPSS, Chicago, IL, USA). Student's t-test was used for a comparison

Results

Analysis of apoptotic cells with flow cytometry

According to our results and other previous published researches, 15, 50 and 100 μM of resveratrol were used for apoptosis induction of the CCRF-CEM cells. After 24 and 48 hours, the apoptosis induction level was evaluated in compare with control cells. The cells, treated by ethanol, were considered as a vehicle control. To examine apoptosis, CCRF-CEM cells were treated with resveratrol, stained with Annexin V and PI and evaluated by flow cytometry assays. As expected, resveratrol induced apoptosis in CCRF-CEM cells in a time and

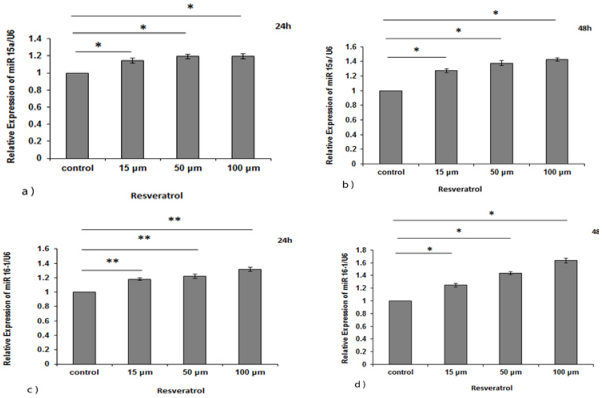

Figure 4. Evaluation of resveratrol effect on miR 15a and miR 16-1 gene expression level by means of RT PCR. CCRF-CEM cells were grown, treated with resveratrol (15, 50 and 100 μM). The expression levels of the indicated miRNAs were examined in CCRF-CEM cells after 24 and 48 hours resveratrol treatment. RNU6 used as an invariant control for evaluation of relative expression of both genes. (a) The miR 15a expression level in CCRF-CEM cells after 24 hours resveratrol treatment. (b) The miR 15a expression level in CCRF-CEM cells after 48hours resveratrol treatment. (c) The miR 16-1 expression level in CCRF-CEM cells after 24 hours resveratrol treatment. (d) The miR 16-1 expression level in CCRF-CEM cells after 48h resveratrol treatment. (*P<0.05, **P<0.01)

dose-dependent manner. Concentrations of 15, 50 and 100 μM increased the amount of apoptosis up to 4.09 % 9.87 % and 23.82 % respectively after 24 hours (Figure 1).

The amount of apoptosis induction after 48 hours treatment with mentioned concentrations of resveratrol were as follow: The concentration of 15 μM resveratrol increased the apoptosis up to 8.44 %.The concentration of 50 μM resveratrol increased the apoptosis up to 26.29 %. The concentration of 100 μM resveratrol increased the apoptosis up to 32.23% (Figure 2). As illustrated in Figure 3 cells undergo dose-dependent apoptotic cell death in response to resveratrol. There was statistically significant in apoptosis increasing between each concentration in different times (Figure 3a, 3b).

The effect of Resveratrol on the expression of miR 15a and mir16-1

To evaluate resveratrol change in miR 15a and miR 16-1 expression in CCRF-CEM cells, which constitute an *in vitro* model of human acute lymphoblastic leukemia, we employed real time PCR analysis to compare resveratrol-treated and untreated cells. MiR 15a and miR 16-1 expression were up-regulated after 24 and 48 hours resveratrol treatment. Resveratrol induced expression of these miRNAs in a time and dose-dependent manner (Figure 4).These results indicate that the resveratrol-induced apoptosis effects were mediated by tumour suppressive miRNAs such as miR 15a and miR 16-1 up-regulation.

Discussion

Nowadays, there are several approaches for treatment of cancer which, surgery radio therapy and chemotherapy are the most common approaches. Regarding the drug resistance, sensitizing the malignant cells to therapeutic agents has turned out to be one of the prevailing approaches of fighting against cancers (Subash et al., 2011). Despite the novel invasive therapy strategies, Resistance to apoptosis partly can be reason of therapy resistance that is becoming a serious problem in anti leukemia therapies. Therefore, induction of apoptosis is considered as a key mechanism for the drugs to take effect. Resveratrol is a natural phytoalexin that has a variety of biological effects on cancerous cells such as anti-inflammatory, anti-proliferative and chemo preventive effects (Cecchinato et al., 2007). It is illustrated that resveratrol can make the resistant cancerous cells sensitive to therapy through different mechanisms; these mechanisms are: modulation of STAT3 expression by the decreasing in the function of tyrosine kinase Src and controlling the induction of survivin (target gene of STAT3) and reinforcing the operation of p53 and production of NO (Hsieh et al., 1999; Burdelya et al., 2005; Niu et al., 2005), modulation of the NF-kB pathway and caspases inducing apoptosis (Estrov et al., 2003), increasing the expression of CD95L and death receptors mediation apoptosis (Marie et al., 1998). In the current study, the effects of Resveratrol were evaluated on the expression of miR 16-1 and miR 15a, in the CCRF-CEM cells and also apoptosis induction were evaluated in this cell line. Clement et al. showed that, resveratrol leads to induction of apoptosis in HL60 cells by the mediation of death receptor (Clement et al., 1998). Also, Surl et al. reported the apoptosis induction in HL60 cells with resveratrol (Surh et al., 1999). Tsan et al. have shown that the apoptosis induced in different pathways without the mediation of death receptors (Tsan et al., 2000). On the other hand, other studies showed that, HL60 and THP1 became resistant to death receptor mediated apoptosis by Resveratrol (Dirks et al., 1997; Della et al., 1998). Bernhard et al. (2000) reported that apoptosis induction by Resveratrol in the Acute Lymphoblastic Leukemia cell lines such as: CEM-C7H2 and Jurkat is independent from Fas/FasL pathway (Bernhard et al., 2000). In the previous studies, it was observed that Resveratrol increases cytoplasmic Cytochrome C (Cytc) of the THP1 cells, which are likely to be released from mitochondria. This event suggests that, apoptosis induction of resveratrol is likely to be carried out through a mitochondria pathway (Dirks et al., 1997). In other research also studied on these two cell lines, increased Bax/Bcl-2 ratio was found, suggesting the pro-apoptotic effect of resveratrol through influencing the intrinsic pathway (Fouad et al., 2013). In our research we demonstrated that, resveratrol induces apoptosis in the CCRF-CEM cells in a time and dose-dependent manner. This effect in 100 μM, is higher in compare with 15 and 50 μM doses. Moreover, time is an effective parameter in induction of apoptosis by Resveratrol, that is, its effect in 48h is more than 24 h. The most important target gens of miR 16-1, miR 15a is mRNA gene of BLC2 that increased in expression level in some of the leukemic cancers (Amelia et al., 2005; Calin et al., 2008). These miRNAs have other targets such as CCND3, CCND1, CCNE1 and CDK6 which take part in cell cycle arrest by means of resveratrol (Cui et al., 2007). Keitaro and colleagues presented that resveratrol increases the tumor suppressing miRNAs by means of increasing the expressing of argonaute protein (Keitaro et al., 2012). The result of our study raises this issue that resveratrol induced miR 15a and miR 16-1 expression based on dose and time dependent manner and increased level of these two miRNAs might have a role in apoptosis induction or predisposition. These results indicate that the resveratrol anti-cancer activities were mediated by miR 16-1 and miR 15a up-regulation that belong to tumour suppressive miRNAs.

The result of our study raises this issue that resveratrol induces apoptosis in a time and resveratrol dependent manner in CCRF-CEM cells. Also, increased expression level of miR 16-1 and miR 15a by means of resveratrol in CCRF-CEM cells might have a role in apoptosis induction and predisposition. Taken together, our study emphasizes that transcriptional induction of miR 15a and miR 16-1 might contribute to resveratrol-induced apoptosis in CCRF-CEM cells. According to our results resveratrol can be regarded as a dietary supplement to improve efficacy of anti-leukemia therapies.

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