Original Article

Resveratrol induces apoptosis in K562 cells via the regulation of mitochondrial signaling pathways

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Abstract: Resveratrol, an edible polyphenolic phytoalexin obtained primarily from root extracts of the oriental plant, Polygonum cuspidatum and from grapes and red wine, has been reported as an anticancer compound against several types of cancer, the accurate molecular mechanisms of by which it induces apoptosis are limited. In the present study, the molecular mechanisms of resveratrol on human leukemia K562 cells apoptosis was examined. Our results showed that resveratrol significantly decreased cell viability and triggered cell apoptosis in K562 cells. Resveratrol-induced apoptosis of K562 cells was associated with the dissipation of mitochondrial membrane potential (MMP) and the release of cytochrome c into the cytosol. Furthermore, the up-regulation of Bax/Bcl-2 ratio, the activation of caspase-3 and increased cleaved PARP was also observed in K562 cells treated with resveratrol. Thus, we considered that the resveratrol-induced apoptosis of K562 cells might be mediated through the mitochondria pathway, which gives the rationale for in vivo studies on the utilization of resveratrol as a potential cancer therapeutic compound.

Keywords: Resveratrol, human leukemia, mitochondrial signaling pathway, apoptosis

Introduction

Leukemia is a clonal disorder with blocked normal differentiation and cell death of hematopoietic progenitor cells. Chronic myelogenous leukemia (CML), a cancer of the white blood cells characterized by the clonal expansion of myeloid precursors, is a myeloproliferative syndrome linked to a hematopoietic stem cell disorder leading to the increased production of granulocytes at all stages of differentiation [1, 2]. The development of tyrosine kinase inhibitors (TKIs) has led to extended lifespans for many patients with chronic myelogenous leukemia. The success of various generations of tyrosine kinase inhibitors in chronic myelogenous leukemia (CML) is well-known, with many patients experiencing long-term benefits from treatment. However, not every patient with CML can tolerate this therapy, shows response to initial treatment, or avoids disease progression or drug resistance, 20% to 30% of patients fail to respond, respond suboptimally, or experience disease relapse after treatment with imatinib, and a key factor is drug resistance [3-6].

A promising source of therapeutic agents is traditional medicine derived from natural compounds. A wide variety of natural compounds derived from medicinal plants have been extensively studied for the treatment of human disease including different types of cancer. Numerous studies have demonstrated that naturally occurring compounds in the human diet may have lower toxicity and less possibility of drug resistance and have long lasting beneficial effects on human health, for example, long-term moderate consumption of red wine is associated with a reduced risk of developing lifestyle-related diseases such as cardiovascular disease and cancer [7-10]. Resveratrol (RSV), trans-3,4', 5-trihydroxystilbene, is a compound obtained primarily from root extracts of the oriental plant, Polygonum cuspidatum and from red grapes [11]. It has been identified that resveratrol has a strong chemopreventive effect against the development of several cancers [12-14]. It is believed that targeting apoptosis in cancer is feasible. However, the molecular signaling mechanisms by which resveratrol exerts its anti-leukemic effects in CML cell lines
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remains incompletely understood. The present study attempts to determine the pro-apoptotic effect of resveratrol and to elucidate the effect of resveratrol on apoptosis involving in the collapse of mitochondrial function in the human CML K562 cell line.

**Materials and methods**

**Drugs**

Resveratrol (Sigma-Aldrich, Inc., St. Louis, Mo, USA) was dissolved in DMSO at 40 mM as a stock solution. The dilutions of all reagents were freshly prepared before experiment.

**Cell lines**

The human myeloid leukemia cell line K562 was purchased from Cell Bank, China Academy of Sciences (Shanghai, China). Cancer cells were maintained in RPMI-1640 (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO), penicillin-streptomycin (100 IU/ml to 100 μg/ml), 2 mM glutamine, and 10 mM HEPES buffer at 37°C in a humidified atmosphere (5% CO₂-95% air).

**Growth and cell proliferation analysis**

The proliferation of gastric adenocarcinoma cells was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. K562 cells (5×10³ per well) seeded in 96-well plates were incubated with increasing concentrations (10, 20, 40, 80, 160 μM) of resveratrol for 24, 48 and 72 h, respectively. The controls were treated with an equal volume of the drug’s vehicle DMSO, but the applied concentration did not exhibit a modulating effect on cell growth. Thereafter, cell growth inhibition was evaluated by MTT assay.

**Hoechst 33258 staining**

K562 cells at the logarithmic-growth phase were seeded into 96-well plates (1×10⁴/well). The cells were cultured in normal medium (control group) or with increasing concentrations of resveratrol (20 μM and 40 μM) for 24 h. Then, the cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature, then washed and stained with Hoechst 33258 (Sigma Aldrich) for 30 min at 37°C. Cells were observed under a Nikon 80i fluorescence microscope equipped with a UV filter (Nikon Corporation, Tokyo, Japan).

**Annexin V/FITC and 7-AAD staining analysis**

K562 cells seeded in 6-well plates (1.5×10⁵ per well) were treated with increasing concentrations of resveratrol for 24 h. Cells were harvested and washed with cold PBS. The cell surface phosphatidylserine in apoptotic cells was quantitatively estimated by using Annexin V/FITC and 7-AAD apoptosis detection kit according to manufacturer’s instructions (Roche, USA). Cell apoptosis was analyzed on a FACScan flow cytometry (Becton Dickinson, USA). Triplicate experiments with replicate samples were performed.

**Mitochondria membrane permeability assay**

The mitochondria membrane potential (MMP) was analyzed by using a JC-1 (5, 5', 6, 6'-tetraethylrhodamine-1, 1', 3, 3'-tetraethylrhodaminecarbocyanine iodide) fluorescence probe kit (Beyotime, China). Briefly, K562 cells cultured in six-well plates exposed to 20 and 40 μM resveratrol for 24 h and then were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

**Preparation of total, mitochondria and cytosol proteins**

Cells in different groups were lysed for total proteins in lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 protease inhibitor mixture (Sigma-Aldrich, USA).

Mitochondria/cytosol kit (Beyotime, China) was used to isolate mitochondria and cytosol according to the manufacturer’s protocol. After washing with cold PBS, cancer cells (5×10⁷) were suspended in 500 μl of isolation buffer containing protease inhibitors and lysed on ice.
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for 10 min. Cells were mechanically homogenized with Dunce grinder. The unbroken cells, debris and nuclei were discarded by centrifugation at 800 g for 10 min at 4°C. The supernatants were centrifuged at 12,000 g for 15 min at 4°C. The supernatant cytosol was collected and pellet fraction mitochondria were dissolved in 50 μl of lysis buffer.

Western blotting assay

Western blotting assay was performed to analyze the expressions of apoptotic and related mitochondrial molecules in K562 cells. Briefly, K562 cells (3×10⁵) seeded in 6-well plates were exposed to various concentrations of resveratrol for 72 h. The cells were harvested and lysates (50 μg of protein per lane) were fractionated by 10% SDS-PAGE as described below. The proteins were electro-transferred onto PVDF membranes, and then incubated with primary antibodies overnight including anti-cytochrome c (4280, Cell Signaling), anti-caspase-3 (9662, Cell Signaling), anti-cleaved PARP (9662, Cell Signaling), anti-Bcl-2 (2772, Cell Signaling), anti-Bax (2872, Cell Signaling), and anti-β-actin (ab6276, Abcam). Appropriate horseradish peroxidase-conjugated secondary antibodies were added in TBST containing 5% BSA. The bound antibodies were visualized by using an enhanced chemiluminescence reagent (Millipore, USA) and quantified by densitometry using ChemiDoc XRS + image analyzer (Bio-Rad, USA) adjusted with β-actin as loading control. Triplicate experiments with triplicate samples were performed.

Statistical analysis

All data were described as mean ± S.D., and analyzed by one-way ANOVA using SPSS/Win11.0 software (SPSS Inc., Chicago, IL.). A p value less than 0.05 was considered statistically significant.

Results

Inhibition of human leukemia cell proliferation

K562 cells treated with resveratrol for 24 h, 48 h, and 72 h were subjected to MTT assay. Our results showed that resveratrol effectively inhibited the proliferation of K562 cells. As shown in Figure 1, the inhibition rate increased from 5.2% to 60.9% after treatment with resveratrol for 24 h, from 6.2% to 67.9% for 48 h, from 5.9% to 70.3% for 72 h. The maximum inhibition rate of 70.3% was found with use of 160 μM for 72 h treatment. These results indicated that resveratrol had a dose- and time-dependent antiproliferative effect on K562 cells in the range of 10-160 μM for 24 h, 48 h, and 72 h of exposure.

Induction of K562 cell apoptosis by resveratrol

To evaluate the resveratrol-induced cell apoptosis of K562 cells, we examined the morphologic changes by Hoechst 33258 staining (Figure 2). The apoptotic morphologic changes in resveratrol-treated groups were observed as compared with the control group. In vehicle control group, nuclei of K562 cells were round and homogeneously stained (Figure 2A), while after treatment of resveratrol the cells revealed significant apoptosis characteristics including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of late apoptotic appearance (Figure 2B, 2C).

Then the apoptosis condition of K562 cells were further analyzed by flow cytometry assay. The results showed evident increase of apo-
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Induction of MMP collapse

The lipophilic and cationic fluorescent dye JC-1 is capable of selectively entering mitochondria, where it forms aggregates and emits red fluorescence when MMP is high. At low MMP, JC-1 cannot enter into mitochondria and forms monomers emitting green fluorescence. The ratio of green to red fluorescence provides an estimate of changes in MMP. JC-1 fluorescence probe showed that MMP in K562 cells was significantly decreased after resveratrol treatment. As shown in Figure 4A, the red fluorescence of JC-1 was gradually decreased and the green fluorescence was correspondingly increased after resveratrol treatment. At the concentration of 20 and 40 μM, the ratios of green to red fluorescence were significantly increased (P<0.01 vs. vehicle control, Figure 4B). These results indicated the collapse of MMP in K562 cells after treatment with resveratrol.

Detection of mitochondrial apoptosis related proteins

First, the distribution of cytochrome c before and after resveratrol treatment was examined by western blotting assay. Cytochrome c in K562 cells was redistributed after resveratrol treatment. In K562 cells, the level of cytochrome c in mitochondria was significantly decreased by 42.6% and 65.7%, and the levels of cytochrome c in cytosol were increased to 145.3% and 193.7% of control group, respectively (P<0.01 vs. vehicle control, Figure 5A).

Furthermore, we examined the expressions of Bax and Bcl-2 and then analyzed the ratio of...
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Bax/Bcl-2. As shown in Figure 5B, the level of Bax was significantly increased and Bcl-2 was obviously decreased in resveratrol-treated cancer cells. Statistical analysis showed that resveratrol in the range of 5 μM increased the ratio of Bax/Bcl-2 by 232.5% and 315.3% for 20 μM and 40 μM (P<0.01 vs. vehicle control), respectively.

Additionally, we measured the molecular alteration of apoptosis related proteins in resveratrol-treated cells. Resveratrol was found to activate the caspases cascade pathway as demonstrated by the increases of cleaved caspase-3 and cleaved PARP in K562 cells. As shown in Figure 5C, the levels of caspase-3 and cleaved PARP were significantly increased in K562 cells exposure to resveratrol.

Discussion

A number of studies have revealed that resveratrol hits a variety of target molecules and cellular signaling pathways pertinent to normal human physiology and directly applicable to all multicellular organisms to control cell proliferation and maintain tissue homeostasis as well as eliminate harmful or unnecessary cells from an organism in physiological and pathological conditions [19, 20]. In cancer cells, the programmed cell death is disrupted thus resulting in the overgrowth of malignant cells [21]. There are two signaling pathways identified to be involved in apoptosis induction including mitochondria-mediated intrinsic and death receptor-mediated extrinsic pathways, and both pathways ultimately lead to the activation of the executioner caspases-3 via diverse pro-apoptotic signals and finally cell death [22, 23]. The mitochondria are important and central mediators of both apoptosis and regulated necrosis. In the intrinsic apoptotic pathway, the mitochondrial outer membrane permeabilization occurs and cytochrome c is released from mitochondria into cytosol after apoptosis initiation, followed by activation of caspase-9 and caspase-3 and thereby cleavage of cleavage of poly (ADP-ribose) polymerase (PARP), which is a specific substrate for caspase-3 [24-26]. The pathological disease states, and it has attracted increasing attention in recent years because of its potent chemopreventive and anti-tumor effects involved various signaling mechanisms such as apoptosis induction, suppression of invasion and metastasis, increased antioxidant capacity, and sensitization to chemotherapy-triggered apoptosis [15-18]. In this research, we confirmed that resveratrol inhibited the proliferation of K562 cells in a concentration- and time-dependent manner, and the accurate pro-apoptotic and molecular signaling pathways mediated by resveratrol to induce its complex anti-leukemic effects in cancer cells was investigated.
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Resveratrol-induced apoptosis of K562 cells might be mediated through the mitochondria pathway. These results support the potential of resveratrol to be developed as a promising agent for treatment of cancers.

Disclosure of conflict of interest

None.

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