Resveratrol inhibits cell viability and proliferation via apoptotic pathways in ovarian cancer

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Ovarian cancer is the most common female malignancy and the main cause of death from gynecological malignancies. Resveratrol (3,4,5 tri-hydroxystilbene) is a phytoalexin and a polyphenolic compound present in human dietary material such as peanuts, mulberries, grapes and red wine. We demonstrated that resveratrol depressed the proliferation of HO8910PM cells in a dose-dependent and time-dependent manner \textit{in vitro} (\textit{P} < 0.001). Resveratrol not only decreased ovarian tumor volume and weight but also influence the tumor forming time \textit{in vivo} (\textit{P} < 0.001). The cell cycle S phase was arrested in response to resveratrol treatment. Caspases activity were also detected by western-blotting in HO8910PM cells treated with resveratrol. Caspase-3 or/and caspase-9 were highly activated in resveratrol-treated HO8910PM cells or xenograft model (\textit{P} < 0.001). Resveratrol significantly inhibits growth and induces apoptosis in HO8910PM cells both \textit{in vitro} and \textit{in vivo}, suggesting that resveratrol is a potential treatment or supplementary measure of ovarian carcinoma.

Key words: Resveratrol, ovarian cancer, apoptosis, cytochrome C, caspase.

INTRODUCTION

Ovarian cancer is the most common female malignancy and ranks first in mortality (Siegel et al., 2014), with a 5-year survival rate as low as 30% (Liao et al., 2014). Due to the difficulties in early detection, the majority of patients are diagnosed with advanced epithelial ovarian cancer with widespread metastatic disease. The patients with advanced ovarian cancer is hard to treat successfully even with the current multi-modal therapies available. Anticancer therapy has identified as methods able to efficiently inhibit cell growth or commit cells to apoptosis.

Previous studies have indicated that some natural supplements exhibit potent anti-tumor properties and modulate apoptosis, differentiation and cell cycle. Most of these chemotherapeutic agents may cause serious side effects on necessitated high doses, whereas natural
supplements, such as resveratrol (3,4,5-trihydroxystilbene), are not potent cytotoxic compounds. Resveratrol, a polyphenolic compound naturally contained in edible material, such as grape skin, peanuts and red wine, is known for its beneficial effects in various biological processes (Kang et al., 2012). Resveratrol inhibits the initiation phase of tumorigenesis, mediates the anti-inflammatory response and induces cell differentiation by acting as antioxidant, anti-mutagen as well as by inducing phase II drug-metabolizing enzymes (She et al., 2003). Therefore, resveratrol has been considered as an anticancer compound in inhibiting tumor promotion and progression (Rahman et al., 2012).

In this study, the effect of resveratrol on inhibitory ability was investigated in human ovarian cancer. Growth inhibitory effect of resveratrol and cell cycle distribution were confirmed, ovarian cancer cells apoptosis induced by resveratrol was demonstrated, and the anticancer activity of resveratrol was analyzed using nude mice xenograft.

MATERIALS AND METHODS

Cell line and cell culture

Ovarian cancer cell line HO8910PM was obtained from Research Institute of Tumor Hospital (Jiangsu, China). HO8910PM cells were cultured in RPMI1640 medium (HyClone, USA) supplemented with 10% fetal calf serum (FCS) (USA) and 100 U/ml penicillin and 100 U/ml streptomycin (St. Louis, MO, USA) at 37°C in 5% CO2. Cell number was counted using a hemocytometer, and cell viability was determined by 0.5% trypan blue exclusion. About 500 mg/ml solution of resveratrol bought from Sino-herb Biotechnology (Xi’an, China) was prepared by dissolving the powder in dimethyl sulfoxide (Sigma). Cells were performed with different concentration of resveratrol at 37°C and equal amount of dimethyl sulfoxide (0.1%) was added to untreated cells.

Cytotoxicity assay

The effects of resveratrol on cancer cells proliferation were performed. Cell viability was determined using cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan). In brief, cancer cells were seeded into 96-well plates at a density of 1×10^4 (50 μL) per well, then incubated for 24 h at 37°C. Seven different concentrations for resveratrol were prepared (50 μL per well), added to the wells, and each concentration was replicated in 3 wells. After incubation for 72 h at 37°C, cells were incubated with 3 μL CCK-8 for 4 h at 37°C. Then, the absorbance was measured at 450 nm by use of a microplate reader (Thermo). Wells with untreated cells or polyphenolic were used as controls. Growth inhibition curves were plotted as percentages of untreated control cells with respect to the standard curves and half-maximal inhibitory concentrations (IC50) were calculated using graphpad prism 5 software (Version 5.01).

Cell apoptosis

Apoptotic cells were assessed with an Annexin V-FITC kit (Biosea, Beijing, China) by flow cytometry. Cells (5 × 10^5) were harvested and washed with cold PBS and re-suspended in 200 μL binding buffer. Then cells were incubated with 10 μL Annexin-FITC and 5 μL propidium iodide (PI) for 15 min at room temperature in darkness. 300 μL binding buffer was added to the suspension. After an hour, apoptotic cells were observed by fluorescence microscope (OLYMPUS, Tokyo, Japan).

Cell cycle analysis

HO8910PM cells were cultured in the presence of the concentrations of resveratrol (60 μM) for 24 h or 48 h. Treated cells were centrifuged and suspended in cold PBS, fixed with 70% cold ethanol at 4°C overnight. In order to remove ethanol, cells were washed and re-suspended in 1 ml PBS. Then, cells were incubated with 0.25 mg/mL RNase A at 37°C for 30 min followed by staining with 400 μL propidium iodide (PI) for 30 min in the dark. Analysis was performed by flow cytometry (Becton- Dickinson FACScalibur).

Animals and xenograft model

Athymic nude mice (BALB/c-nu/nu, female), 4 weeks old and weighing approximately 18 to 20 g were obtained from Vital River Laboratory Animal Technology Co.Ltd (Beijing, China). Animal care and experimental procedures were in accordance with the guidelines on the ethics committee for animal experiments. All the experiments were supported by Center of Experimental Animals in Nanjing University (China). Thirty mice were randomized to three groups (n=10) with low dose group (50 mg/kg resveratrol), high dose group (200 mg/kg resveratrol) and the control (200 mg/kg ethyl alcohol). HO8910PM cells (2×10^6) were suspended in 200 μL saline solution and injected into back of mice. Mice of group one were treated with resveratrol of 50 mg/kg every two days (low) and group two were treated with 200 mg/kg every two days (high), while group three were considered as a control group with equivalent ethyl alcohol at the same time. Then, tumor growth speed and tumor size were examined for 8 weeks. The growth of primary tumors was monitored every 72 h by measuring tumor diameters with calipers and calculating tumor volume using the formula: Volume=Width^2×Length/2. Tumors were weighed and collected after slaughter. Pathological changes in tumor xenograft excised from nude mice were evaluated by a professional clinician. Samples of these xenografts were fixed in 10% neutral buffered formalin for 24 h, dehydrated, embedded in paraffin wax, and sectioned (3 to 5 μm). The sections were stained by hematoxylin and eosin (HE). Protein extracts of tumor were obtained for western-blotting.

Western-blotting

HO8910PM cells (1×10^6) were treated with resveratrol (60 μM) and collected at 24 h after the incubation. The total samples were lysed in RIPA lysis buffer (Beyotime, China) supplemented with 10 mM PMSF on ice. The lysate was centrifuged at 12000 rpm for 15 min at 4°C. 50 μg of total proteins were electrophoresed in 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked in 1% BSA and were incubated with antibodies at 4°C overnight. Purified mouse anti-cytochrome c, anti-caspase-3, anti-caspase-9 and anti-α-Tublin monoclonal antibodies were from Cell Signal technology (USA) and used as primary antibodies (1:2,000). α-Tublin was used as reference for the experimental data analysis.

Statistical analysis

All data were presented as mean ± standard deviation (SD) in
The inhibitory effects of resveratrol on HO8910PM cells in cytotoxicity assays were assessed. As shown by the inhibition curves in Figure 1A, HO8910PM was a sensitive cell line to resveratrol with the IC\textsubscript{50} value of 20 μM ($P < 0.001$). Moreover, compared with the control, when HO8910PM cells were treated with the afore-mentioned IC\textsubscript{50} concentrations of resveratrol for different time lengths (24 and 48 h), the inhibitory rates of HO8910PM cells increased with prolongation of treatment times ($P < 0.001$). The maximum inhibition reached 76% at 48 h, after HO8910PM cells were treated with 60 μM resveratrol. These results revealed that resveratrol depressed the proliferation of HO8910PM cells in a dose-dependent and time-dependent manner.

**RESULTS**

**The characteristics of resveratrol against cancer cells**

The inhibitory effects of resveratrol on HO8910PM cells in cytotoxicity assays were assessed. As shown by the inhibition curves in Figure 1A, HO8910PM was a sensitive cell line to resveratrol with the IC\textsubscript{50} value of 20 μM ($P < 0.001$). Moreover, compared with the control, when HO8910PM cells were treated with the afore-mentioned IC\textsubscript{50} concentrations of resveratrol for different time lengths (24 and 48 h), the inhibitory rates of HO8910PM cells increased with prolongation of treatment times ($P < 0.001$). The maximum inhibition reached 76% at 48 h, after HO8910PM cells were treated with 60 μM resveratrol. These results revealed that resveratrol depressed the proliferation of HO8910PM cells in a dose-dependent and time-dependent manner.

**Resveratrol promoted condensable chromatin in HO8910PM**

HO8910PM cells were treated for 24 h with 60 μM resveratrol and then utilized for cytofluorimetric analyses upon staining with annexin V-FITC (Figure 1C). Fluorescence microscope analysis of nuclei upon staining with DNA-specific dye Hoechst 33342, showed that HO8910PM cells treated with 60 μM resveratrol for 48 h, exhibited areas of blebbing, cell shrinkage and condensed chromatin, suggesting that resveratrol induced HO8910PM cells apoptosis.
Resveratrol arrested cell cycle progression in HO8910PM

The cell cycle distribution of HO8910PM cells was analyzed after treatment with 60 μM of resveratrol for 24 and 48 h. As shown in Figure 2, compared with the control, the percentage of the cell population in the G1 phase was decreased at 24 h or 48 h ($P < 0.001$), while the S population was dramatically increased at 24 h or 48 h ($P < 0.001$), suggesting that cell cycle S phase was arrested in response to resveratrol treatment. However, little change was observed in the G2 phase of resveratrol-treated or polyphenolic-treated HO8910PM cells, except at 48 h ($P < 0.001$). In addition, the cell population in G1 or S phase was continued to decrease or increase by resveratrol treatment in a time-dependent manner, indicating that resveratrol induced inhibition of HO8910PM cells, predominantly through cell cycle arrest.

The effect of resveratrol on nude mice xenograft

Resveratrol was then performed in vivo antitumor evaluation in BALB/c nude mice and HO8910PM tumor xenograft model. Nude mice (30) were separated randomly into three groups (low resveratrol, high
Figure 3. *In vivo* anticancer activity of resveratrol in mice bearing HO8910PM tumor xenograft. (A) Effect of low resveratrol (50 mg/kg), high resveratrol (200 mg/kg), and ethyl alcohol (200 mg/kg) on growth of tumor xenograft. Tumor growth was tracked by the mean tumor volume (mm³) ±SD (n = 10). ***P < 0.001, P versus the control. (B) Tumor forming time was calculated when tumor size reaches to about 100 mm³. ***P < 0.001, P versus the control. (C) Tumor weight was recorded after the mice were executed with CO₂. ***P < 0.001, P versus the control. (D) Photographs of tumors from the treatment group and the control group. Group 1: Ethyl alcohol was used as a control. Group 2: Mice were treated with resveratrol of 50 mg/kg every two days. Group 3: Mice were treated with resveratrol of 200 mg/kg every two days. (E) The pathological section of tumor in Nude Mice. The animals were executed with CO₂ after 8 weeks treatment and pathological section of tumor were made to observe the minute structure. Group 1: Ethyl alcohol was used as a control. Group 2: Mice were treated with resveratrol of 50 mg/kg every two days. Group 3: Mice were treated with resveratrol of 200 mg/kg every two days.

Resveratrol, and the ethyl alcohol groups). After HO8910PM cells with matrigel were inoculated subcutaneously in the back of mice, the treatments were performed. When the implanted tumor grew up to about 100 mm³, tumor forming time was calculated. The tumor volumes were measured weekly. As shown in Figure 3A and B, comparison to the mice in the polyphenolic group, both low and high resveratrol groups experienced significant reductions in tumor volume (P < 0.001). Furthermore, the tumor forming time was 124.6±7.1 h and 176.4±2.4 h in the group of low and high resveratrol, respectively, while 72.6±6.1 h in control group (P < 0.001).

Tumor weights were recorded after slaughter on treatment for 56 days, and the inhibitory rates on tumor growth are as shown in Figure 3C and D. Resveratrol
possessed significant antitumor activity in the HO8910PM tumor xenograft model with an inhibitory rate of 45.7 and 71.7% in low and high resveratrol groups, respectively ($P < 0.001$). The higher concentration of resveratrol, the lower tumor weight and size existed, suggesting that resveratrol exhibits more potent antitumor activity in vivo.

The effect of resveratrol on tissues in the xenograft model was also observed. Using transmission electron microscope (TEM), it was found that cells in experimental group showed the phenomenon of karyopyknosis, mitochondria swelled and decrease in cellular junction (Figure 3E). These kinds of phenomenon were more intensified under the condition of the higher concentration of resveratrol, indicating the effect of resveratrol on tumor growth in vivo.

**Resveratrol up-regulated caspases 9 and 3 expression**

The apoptosis regulating factors were detected by western-blotting using resveratrol-treated HO8910PM cell lysate at 48 h (Figure 4A and C). Compared with the control, the caspase-3 and cytochrome c were highly activated in HO8910PM cells treated with resveratrol ($P < 0.001$), suggesting that the apoptosis was irreversible, and the caspase apoptosis pathway was activated by the resveratrol treatment. Moreover, these factors were also detected by western-blotting using protein extracts of tumor in xenograft model (Figure 4B and D). Compared with the control group, the expression levels of caspase-3, caspase-9 and cytochrome c were obviously increased in high dose groups at the concentration of 200 mg/kg resveratrol ($P < 0.001$). The results corresponded with annexin V-FITC staining, indicating that the resveratrol induced HO8910PM cells apoptosis.

**DISCUSSION**

Resveratrol has been considered as a potential anticancer agent that displays pleiotropic effects and has a multitude of biochemical and molecular actions, including inhibition of free radical formation and activities of cyclooxygenase (COX), inducible nitric oxide synthase, cytochrome P-450 and protein kinase C. Resveratrol could also induce apoptosis of various cancer cells, such as breast cancer, neuroblastoma and gastric cancer (Ren et al., 2015; Alayev et al., 2015; Liu et al., 2014). It might inhibit each phase of tumor growth by interacting with
several cellular targets, thereby activating cell cycle arrest and death (Colin et al., 2006; Meehyun et al., 2009; Hsieh et al., 1999). This study investigated the effects of resveratrol on ovarian cancer cells using HO8910PM cells in vitro and in vivo. According to Vergara et al. (2012), resveratrol inhibited ovarian cancer cells in vitro by mediating a variety of cellular pathways. In the present study, it was concluded that resveratrol is able to inhibit HO8910PM cells growth with an IC_{50} value of 20 μM (Figure 1A). Meanwhile, resveratrol depressed the proliferation of HO8910PM cells in a dose-dependent and time-dependent manner. In particular, at concentration of 60 μM, the percentages of inhibitory cells reach about 50 and 76% at 24 and 48 h, respectively (Figure 1B). These finding is also consistent with that of Baribeau et al. (2014), who found that resveratrol suppressed the proliferation of ovarian cancer cell lines A2780 and SKOV3 in a high concentration. Resveratrol has also displayed anti-cancer activity in vivo in a dose-dependent manner (Figure 3A). Resveratrol does not only decrease ovarian tumor volume and weight but also influence the tumor forming time (Figure 3B and C). Compared with the control, the tumor forming time was significantly lengthened, whereas the tumor weight was severely decreased in groups of low and high resveratrol concentration, respectively (P < 0.001).

Several reports showed that resveratrol decreased the proliferation and induced apoptosis of cancer cells through diverse mechanisms, among which, the most important one is altering the cell cycle distribution (Frazzi et al., 2013; Casanova et al., 2012; Bai et al., 2010). In the present study, marked S phase arrest and apoptosis with distinct growth inhibition were observed in HO8910PM cells treated with the concentration of 60 μM resveratrol (P < 0.001) (Figure 2). Resveratrol decreased the percentage of cells undergoing G1 phase as well as increased the percentage of cells in S phase, suggesting that an increase in the percentage of HO8910PM cells in S phase result in a more sensitivity to resveratrol. This result is in agreement with the previous findings that resveratrol has been shown to induce cell cycle arrest in G1/S in a variety of other human cancer cell lines (Liu et al., 2014).

Caspases are important proteolytic enzymes that play critical roles in necrosis, inflammation, and apoptosis (Salvesen and Riedl, 2008). The activations of these caspases lead to irreversible morphological or biochemical changes in cells. The activation of caspase-3 is a very rapid process in the cell death process (Tyas et al., 2000). Activated caspase-9 ultimately triggers the subsequent cleavage of caspase-3 to form active caspase-3, thus leading to apoptosis (Kim et al., 2012; Bao and Shi, 2007; Prabhu et al., 2013). Cytochrome c, an apoptogenic protein, might form an apoptosome complex, which activated a downstream caspase-9 (Kim et al., 2012; Roslie et al., 2012). The present results revealed that the expression of caspase-3 was obviously increased in HO8910PM cells treated with resveratrol (P < 0.001). Meanwhile, the expression levels of caspase-9 were significantly increasing at the concentration of 50 and 200 mg/kg resveratrol in tissues lysate from HO8910PM xenograft model (P < 0.001). These results indicated that resveratrol promotes apoptosis in ovarian cancer cells, which eventually activated downstream caspase-9 and caspase-3 to execute the apoptotic process.

A decrease in a viability of colorectal and bladder cancer cells treated with resveratrol was associated with its ability to induce apoptosis (Liu et al., 2014; Wu et al., 2014). In present study, HO8910PM cells were treated with resveratrol and then utilized for cytofluorimetric analyses. As shown in Figure 1C, several biochemical phenomena were observed, including blebbing, cell shrinkage and chromatin condensation which were considered as features of apoptosis. Especially, similar phenomena existed in tissues slices from HO8910PM xenograft of resveratrol treatments. After the animals were executed with CO2 at 8 weeks treatments, the pathological sections of tumor were made to observe the minute structures. The changes of pathology were obvious in cellular junction, such as karyopyknosis, mitochondria swelled and decreased. Consistent with a previous result, the apoptosis was detected by western-blotting in HO8910PM cells after resveratrol treatment at 48 h.

In conclusion, resveratrol significantly inhibits growth and induces apoptosis in HO8910PM cells both in vitro and in vivo. The inhibition in vitro and the anticancer activity in vivo of resveratrol against HO8910PM cells mainly are attributed to apoptosis via a caspase pathway by caspase-3 and caspase-9 activation. Thereby, these data clarify the anti-cancer effect of resveratrol on ovarian cancer, suggesting that resveratrol is a potential treatment or supplementary measure of ovarian carcinoma and might provide a scientific basis for improving survival in patients with ovarian cancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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