Original Contribution

Potent antiproliferative effects of resveratrol on human osteosarcoma SJSA1 cells: Novel cellular mechanisms involving the ERKs/p53 cascade

Moussa Alkhalaf *, Sahar Jaffal

Department of Biochemistry, Faculty of Medicine, Kuwait University, Safat 13110, Kuwait

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Abstract

The chemopreventive activity of resveratrol (RSVL) has been demonstrated in several types of cancer. However, its effects and the underling mechanisms remain poorly understood. In this study, we investigated the involvement of the mitogen activated protein kinase (MAPK)/p53 signal transduction mechanism in RSVL-induced growth inhibition using a human osteosarcoma cell line. We demonstrate that RSVL reduces cell viability and growth of SJSA1 osteosarcoma cells. Morphological profiles and 4,6-diamidino-2-phenylindole nuclear staining of RSVL-treated cells indicated marked nuclear fragmentation. Cleavage of the (116-kDa) poly(ADP-ribose) polymerase protein into an 89-kDa fragment (a proapoptotic marker system) was substantially augmented by RSVL treatment. RSVL-dependent growth impairment was preceded by enhanced phosphorylation of extracellular signal-regulated kinase (ERK)1/2 (at Thr202/Tyr204). Likewise, RSVL increased the phosphorylation of p53 tumor suppressor protein (at Ser15). The effects of RSVL on ERKs and on p53 phosphorylation were abrogated by either the MAPK inhibitor PD98059 or the p53 inhibitor pifithrine-α. The present study indicates that RSVL antiproliferative effects on osteosarcoma cells are mediated by the activation of the ERKs/p53 signaling pathway and therefore identifies new targets for strategies to treat and/or prevent osteosarcoma. © 2006 Elsevier Inc. All rights reserved.

Introduction

Resveratrol (trans-3,4′,5-trihydroxystilbene, RSVL) is a natural phytoalexin found in grapes, berries, and red wine [1]. It has been reported to confer protection against some cardiovascular diseases, a dogma that is commonly designated as “the French paradox of red wine” [2,3]. Equally important, but later discovered, was the cancer chemopreventive activity for RSVL. Studies revealed that RSVL is capable of inhibiting the three major stages of carcinogenesis, namely tumor initiation, promotion, and progression [4]. These effects are mediated through inhibition of enzymes involved in cell proliferation such as DNA polymerase and ribonucleotide reductase, which is involved in S-phase entry [5,6], or activation of proteins involved in apoptosis/growth arrest such as the cAMP/kinase-A cascade [7]. RSVL is a growth inhibitory for several human cancer cell lines such as HL60 promyelocytic leukemia cells [8], JB6 mouse epidermal cells [9], CaCo-2 colorectal cells [10,11], A431 epidermoid carcinoma cells [12], and the breast-cancer cells MCF7 [13], MDA-MB-231 [14,15], KPL-1 and MKL-F [16], and T47D cell lines [17] in addition to animal models of carcinogenesis [4,18,19]. In this study we have evaluated the chemopreventive action of RSVL on the human osteosarcoma cell line SJSA1. Because a potent growth inhibitory effect was evident, we further checked the cellular and molecular mechanisms whereby RSVL induced these effects. We examined the involvement of the MAPK/p53 pathway in RSVL-induced growth inhibition in these cells.

Materials and methods

Abbreviations: resveratrol, RSVL; FBS, fetal bovine serum; DAPI, 4,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; ECL, enhanced chemiluminescence.

⁎ Corresponding author. Fax: +965 5338908.
E-mail address: alkhalf@hsc.edu.kw (M. Alkhalaf).

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USA). p44/42 MAP kinase, phospho-p53 (Ser15), poly (ADP-ribose) polymerase (PARP) polyclonal antibodies and phospho-p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). MAPK inhibitor PD98059 and p53 inhibitor pifithrine-α were obtained from Calbiochem (Ontario, Canada). All cell culture reagents were obtained from Gibco-BRL (Paisley, UK).

Cells and culture conditions

The human osteosarcoma cell line SJSA1 was kindly provided by Bohdan Wasylyk (IGBMC core facility, Strasbourg, France). The cells were maintained in RPMI1640 medium (Gibco BRL) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 incubator at 37 °C. The cells were passaged twice a week by harvesting with trypsin/EDTA and seeding into 25-cm² flasks.

Cell proliferation test

To study the effect of RSVL on cell proliferation, cells were seeded in six-well tissue culture plates at approximately 10⁴ cells/well in the presence of 10% FBS for 24 h. The effects of RSVL alone or with inhibitors were tested in media containing low serum concentration (0.5%) and containing high serum concentration (10%). After 3 days of treatment with RSVL or inhibitors, the cells were harvested by treatment with trypsin/EDTA and counted with a Coulter counter (Type Z2).

Morphologic analysis

Cells were seeded in six-well tissue culture plates at approximately 10⁴ cells/well in the presence of 10% FBS for 24 h. The medium was then changed using fresh RPMI-1640 supplemented with 0.5% FBS with vehicle (control) or with 100 μM RSVL. Following 48 h of incubation, the cells were examined with a Leitz phase-contrast microscope (Labovert FS) with low power magnification (×20), and they were photographed by a Leica Camera (Wild MPS52).

Cell viability analysis with trypan blue dye exclusion test

Cells were seeded in twelve-well tissue culture plates at approximately 10⁴ cells/well in the presence of 10% FBS for 24 h. The effect of RSVL was tested in medium containing low serum concentration (0.5%) and containing high serum concentration (10%). After 3 days of treatment with RSVL or inhibitors, the cells were harvested by treatment with trypsin and counted with a Coulter counter (Type Z2). (A) RSVL significantly inhibited the growth of SJSA1 cells in both high-FBS (10%) and low-FBS (0.5%) media. The inhibition was observed at 1 × 10⁻¹ to 100 μM RSVL. Means ± SD from triplicate experiments are presented. * Significantly different from control (p < 0.001). (B) RSVL reduces SA1 cell viability. Cell viability determined by trypan blue exclusion is reduced in a dose-dependent manner in SA1 cells treated for 48 h with the indicated concentrations of RSVL in the presence of 0.5% FBS.

Nuclear DNA fragmentation analysis using DAPI staining

To identify the cells undergoing apoptosis we used the 4,6-diamidino-2-phenylindol staining method. Cells were seeded at 1 × 10⁴ into 35-mm multiwell plates containing RPMI1640 medium supplemented with 10% FBS and incubated for 24 h. The cells were then treated with vehicle or with 100 μM RSVL for 48 h in the presence of 0.5% FBS. Cells were washed with PBS, fixed with 100% methanol, and then
stained with DAPI (1 μg/ml). The stained nuclei were visualized under a Zeiss Axioplan microscope equipped with DAPI filter using a 40× oil objective. Photographs were taken with Kodak TMax 400 film. Condensed or fragmented nuclei were scored as apoptotic cells.

**PARP cleavage assay**

Cells (5 × 10^6) were plated into 75-cm² cell culture flasks in the presence of 10% FBS for 24 hours. Cells were then placed in 0.5% FBS-supplemented growth medium for 24 h prior to treatment. Cells were treated for 24 h with vehicle (control), 100 μM RSVL alone, or in combination with the MAPK inhibitor PD98059 (50 μM) or the p53 inhibitor pifithrine-α (70 μM) in the presence of RPMI 1640 medium containing 0.5% FBS. Following harvesting, cell pellets were resuspended in RIPA buffer (see composition below), and total protein extracts were prepared by freezing (in liquid nitrogen) and thawing (at 37 °C) three times in a row, then centrifuged at 14,000g for 10 min and supernatants were saved. Proteins (~50 μg) were separated on 8% SDS–PAGE and transferred to nitrocellulose membranes. The latter were incubated in a blocking solution (4% nonfat dry milk in 0.05% Tween 20 in PBS buffer) and anti-PARP antibody (1:1000) in 2% nonfat dry milk. During apoptosis, intact PARP is specifically cleaved by members of the caspase family of proteases (e.g., caspase-3) into two proteolytic fragments of approximately 25 and 89 kDa. Full-length PARP (116 kDa) and cleaved fragments (89 kDa) were visualized by ECL procedure.

**Western blot analysis**

Cells 5 × 10^6 were plated into 75-cm² cell culture flasks in the presence of 5% FBS for 24 h. Cells were then placed in 0.5% FBS-supplemented growth medium for 24 h prior to treatment. The cells were treated with RSVL or with inhibitors
in 0.5% FBS-supplemented growth medium for the indicated time. At the end of incubation time, cells were washed twice with ice-cold PBS before being scraped off with a rubber policeman and centrifuged to form a pellet. The cell pellets were resuspended in 100–300 μl fresh ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 8, 0.1% SDS (w/v), 0.5% sodium deoxycholate (w/v), 1% Triton X-100 (v/v), 150 mM sodium chloride, 10 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The resuspended pellets were transferred to Eppendorf tubes, snap-frozen in liquid nitrogen for 30 s, and then incubated in a 37 °C water bath for 1 min. Freezing and thawing were repeated three times followed by centrifugation at 13,000g for 5 min. The resulting supernatant was saved and protein concentration was determined by the Bradford method. Extracts were boiled for 3 min in 2× SDS buffer. Equal amounts of protein were loaded on 8% SDS–PAGE according to the method of Laemmli and electrotransferred to nitrocellulose membranes. The blots were incubated with the indicated antibodies at a 1/1000 dilution in 2% nonfat dry milk for 1 h and then incubated with the appropriate peroxidase-conjugated secondary antibodies at a 1/2000 dilution for 1 h. Immunoreactive bands were visualized by incubation with luminol (according to the manufacturer’s instructions; ECL Western blotting detection system from Amersham). Actin was also visualized as loading control using monoclonal antibody (Sigma).

Data are expressed as the mean ± SD. The significance of the difference between the DMSO-treated control cells and RSVL-treated cells was analyzed by Student’s test *t*-test. A value of *p* < 0.05 was considered statistically significant.

**Results**

**RSVL causes dose-dependent growth inhibition and nuclear fragmentation**

To examine the antitumor activity of RSVL in SJSA1 human osteosarcoma cells, we first investigated its effects on cell growth. As can be seen in Fig. 1A, RSVL caused marked dose-dependent growth inhibition. These responses were evident in both high (10%) and low (0.5%) serum media. Statistically significant reduction of cell number (*p* < 0.001) was observed at 10–100 μM RSVL. Parallel cell viability studies, employing the trypan blue exclusion method, showed a similar concentration-dependent inhibition of cell viability by RSVL (Fig. 1B). To further characterize whether the growth inhibitory activity of RSVL in SJSA1 cells was related to the induction of apoptosis, we examined the effect of 100 μM RSVL on cell morphology and on nuclear DNA fragmentation by fluorescent microscopy using the DNA-binding fluorescent dye DAPI. Fig. 2B shows marked decrease in number and aberrant gross morphological changes in RSVL-treated cells; these are dying cells (clearly displayed is the aberrant morphology in cells treated with RSVL as compared to the normal control cells in (Fig. 2A). The presence of many round floating cells was observed also. With
DAPI staining, RSVL (Fig. 2D) induces a nuclear fragmentation pattern as compared with control cells (Fig. 2C), suggesting that these cells are undergoing apoptosis. We examined the capacity of RSVL to stimulate the breakdown of PARP 116 kDa into its 89-kDa fragment, a well-established proapoptotic response. Immunoblot analysis with a specific antibody that recognizes full length PARP and its cleavage product clearly shows an enhanced production of the 89-kDa fragment following treatment with RSVL (100 μM) (Fig. 3A). Thus, these observations support the earlier morphological/staining data showing RSVL to induce apoptosis in SJSA1 cells. To further explore the mechanisms whereby RSVL induces these apoptotic signals, we repeated these PARP experiments in the presence of inhibitors for the p53 protein (pifithrine) and for the MEK/ERK pathway (PD98059). Both inhibitors enhanced PARP cleavage. In addition, both inhibitors showed additive effects on RSVL-induced PARP fragmentation (Fig. 3B).

To further substantiate the significance of these molecular findings and link it with cell growth, we examined the impact of p53- and ERK-inhibitors on RSVL-evoked inhibition of cellular growth. Fig. 4 shows that 50 μM PD98059 significantly reversed the effect of RSVL on cell proliferation. * Data from RSVL combined with MAPK inhibitor-treated samples were significantly different from the RSVL alone ($p < 0.05$).

Fig. 5. Dose-dependent effects of MAPK inhibitor on RSVL-induced growth inhibition. Cell culture and treatment conditions were the same as in Fig. 4. This figure shows that 50 μM PD98059 significantly reversed the effect of RSVL on cell proliferation. * Data from RSVL combined with MAPK inhibitor-treated samples were significantly different from the RSVL alone ($p < 0.05$).

Fig. 6. RSVL induces p44/42 (Thr202/Tyr204) kinase activation. Cells were treated with 100 μM RSVL for the indicated times in the presence of 0.5% FBS, and then western blot analysis was performed using phospho-p44/42 (E10) monoclonal antibody. This antibody detects the phosphorylated (active) forms of ERKs at Thr202/Tyr204 residues or with a actin antibody. (A) RSVL (100 μM) stimulated the phosphorylation of ERK1/2. (B) Graphs represent averaged data, quantified by densitometry and randomized to values of actin expression. Response was evident as early as 30 min. The phosphor-p42 was more phosphorylated by RSVL compared to p-44. Values are means ± SD of three independent experiments.

Fig. 7. MAPK inhibitor decreases RSVL-induced ERKs phosphorylation. Cells were treated with 100 μM RSVL alone or in combination with 50 μM PD98059 MAPK inhibitor for 4 h in the presence of 0.5% FBS and then western blot analysis was performed using phospho-p44/42 (E10) monoclonal antibody or with a polyclonal antibody that detects total ERK44/42. RSVL induced the phosphorylated kinase (top), whereas PD98059 was able to decrease it. However, the decrease did not reach the control level. The total ERKs were not affected by RSVL stimulation (bottom).

Fig. 8. RSVL induces p53 phosphorylation at serine 15. (A) Western blot analysis of extracts from SJSA1 cells treated with 100 μM RSVL for the indicated times in the presence of 0.5% FBS, using phospho-p53 (Ser15) polyclonal antibody or actin monoclonal antibody. (B) Bar graph shows changes in mean band optical density ± SD in three experiments as analyzed by Chemi Genius Syngene (Bioimaging System). Actin levels served as controls for protein loading.
increasing amounts (0.5, 5, and 50 μM) decreases the RSVL-induced growth inhibition (\( p < 0.05 \)), whereas pilifirine-α alone showed an antiproliferative effect and had no additive effect on RSVL-induced growth inhibition.

**RSVL-induced growth inhibition is mediated through the MAPK pathway**

To confirm that the effect of RSVL on cell proliferation and apoptosis was mediated through the MAPK pathway, the cells were treated with 100 μM RSVL alone or in combination with increasing amounts (0.5, 5, and 50 μM) of PD98059 for 3 days in the presence of 0.5% FBS. As shown in Fig. 5, 50 μM inhibitor significantly reversed the effect of RSVL on cell proliferation (\( p < 0.05 \)).

**RSVL phosphorylation of ERKs is linked to activation of the p53 protein**

In view of the ability to induce nuclear fragmentation and PARP cleavage and the effects of inhibitors of ERK/p53 on PARP cleavage, it was of interest to determine whether RSVL can directly activate ERK and the p53 protein. Immunoblot analyses targeting the phosphorylated (active) forms of ERKs at Thr202/Tyr204 residues (Fig. 6) indicate that RSVL enhances activity for both ERKs (p42 and p44). This response was evident as early as 30 min and thereafter also. In another experiment, we demonstrated that RSVL-induced ERK phosphorylation was largely inhibited by 50 μM PD98059 MAPK inhibitor (Fig. 7). Experiments employing antibody against phosphorylserine-15 of the p53 protein (activated form of p53) indicated that RSVL also activated the p53 protein (Fig. 8). This RSVL-induced activation of p53 was sensitive to treatment with the ERK inhibitor PD98059 and p53 inhibitor, thus revealing a causal relationship in the activation of ERK and p53 in response to RSVL treatment (Fig. 9). RSVL had no effects on expression states of both ERKs (Fig. 7) and p53 (data not shown).

**Discussion**

We have previously shown that RSVL-induced growth inhibition of MCF7 human breast cancer cells is mediated through the activation of the cAMP/kinase-A cascade [7]. In the current study we have investigated the possible mechanisms by which RSVL modulates cell growth of cancer cells. We demonstrated that this phytoestrogen compound induced apoptosis in SJSA1 human osteosarcoma cells. We have provided evidence that RSVL-induced apoptosis was associated with the activation of the MAPK/p53 cascade. We have therefore identified potential new mechanisms underlying the antiproliferative properties of this natural drug in human osteosarcoma cells. RSVL is currently being evaluated in preclinical studies as a potential cancer chemoprevention drug. RSVL has previously been shown to exert antitumor properties and to inhibit invasiveness [20]. Osteosarcoma is the most frequent primary malignant tumor of bone with a high propensity for metastasis [21]. Our findings provide further support for the clinical potential of this compound. We have found that osteosarcoma cells were more sensitive to RSVL-induced growth inhibition than T47D, MCF7, and MDA-MB-231 breast cancer cells (unpublished data). RSVL caused a dose-dependant growth inhibition and loss of cell viability. These effects were abrogated by the MAPK inhibitor PD98059, suggesting that RSVL-induced growth inhibition is mediated through the MAPK (ERK1/2) pathway and the activation of the p53 tumor suppressor protein function. This cell line has amplification of the mdm2 gene and therefore inactivated p53 function [22]. Phosphorylation of specific serine residues of p53 has been shown to increase p53 stability and modulate p53-dependent transcription [23]. Phosphorylation of serine-15 occurs in response to DNA damage and mutation in serine-15 impairs the ability of p53 to induce apoptosis [24]. It seems that RSVL induction of the p53 phosphorylation at serine-15 may reestablish the tumor suppressor function of this protein in these cells and may be linked to activation of p53. Our data on the activation of the MAPK/p53 pathway are in agreement with results from several laboratories that demonstrated a p53-dependent pathway in the induction of apoptosis in several systems by RSVL [12,25–29]. She et al [30] demonstrated that RSVL-induced activation of p53 and apoptosis involves c-jun NH(2)-terminal kinases. However, the exact mechanism of p53 activation by RSVL is not well understood. Our data show that RSVL-induced phosphorylation of p53 in these cells was sensitive to treatment with the ERK inhibitor PD98059 and p53 inhibitor, thus revealing a causal relationship in the activation of ERK and p53 in response to RSVL treatment.

The tumor suppressor gene p53 and its protein control critical cellular functions involved in apoptosis and in the control of the cell cycle [31]. Therefore, reestablishing p53 function in these cells in response to resveratrol treatment may lead to the induction of cell cycle arrest, cell differentiation, or apoptosis. It was demonstrated by several authors that the anticancer properties of RSVL were related to its abilities to cause cell cycle arrest in the G1 phase [32] or in the S-G2 phase transition [13,33,34] and to trigger apoptosis in a variety of cancer cell lines [17,35–37]. Thus, the effect of RSVL on cell proliferation is cell type dependent. In other words, RSVL-induced growth inhibition can be caused by cell cycle arrest, cell differentiation, or direct programmed cell death. In this study, we have demonstrated that the phytoalexin RSVL exerted
proapoptotic effects (as shown by the induction of PARP fragmentation) on human osteosarcoma SJSA1 cells. However, this induction of PARP fragmentation was not associated with RSVL stimulation. When RSVL was combined with MAPK or p53 inhibitors, PARP fragmentation was maximal. In these conditions, RSVL had no effects on cell growth. In addition, when used at 100 μM in the presence of low or high serum concentration, RSVL was not able to induce the characteristic apoptotic DNA ladder (data not shown). These results suggested to us that RSVL-induced growth inhibition may be caused by another mechanism. Ragione et al. [38] demonstrated that RSVL-induced growth arrest and differentiation of K562 cells was associated with Waf-1 overexpression. However, to further confirm the direct involvement of the p53 signaling pathway in RSVL-induced growth inhibition or cell differentiation in osteosarcoma cells, p53-responsive genes need to be analyzed in these cells after RSVL treatment.

In conclusion, the present study contributes to insights into the molecular mechanisms of RSVL-induced growth inhibition and, in general, of its chemopreventive activity in cancer models. Our data indicate that RSVL antiproliferative effects on osteosarcoma cells are mediated by the activation of the ERK-p53 signaling pathway and therefore identify new targets for strategies to treat and/or prevent osteosarcoma.

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References


