Resveratrol is a potent inducer of apoptosis in human melanoma cells

Richard M. Niles[a],*, Margaret McFarland[a], Mathew B. Weimer[a], Alka Redkar[b], Ya-Min Fu[b], Gary G. Meadows[b]

[a]*Department of Biochemistry and Molecular Biology, Joan C. Edwards School of Medicine, Marshall University, 1542 Spring Valley Drive, Huntington, WV 25704, USA*[1]
[b]*Cancer Prevention and Research Center, College of Pharmacy, Washington State University, Pullman, WA, USA*[1]

Abstract

Resveratrol is a plant polyphenol found in grapes and red wine. It has been found to have beneficial effects on the cardiovascular system. Resveratrol also inhibits the growth of various tumor cell lines in vitro and inhibits carcinogenesis in vivo. In this study we examined the effect of resveratrol on growth of two human melanoma cell lines. We found that this plant polyphenol inhibited growth and induced apoptosis in both cell lines, with the amelanotic cell line A375 being more sensitive. The potential involvement of different MAP kinases in the action of resveratrol was also examined. Although resveratrol did not alter the phosphorylation of p38 or JNK MAP kinases in either cell line, it induced phosphorylation of ERK1/2 in A375, but not in SK-mel28 cells. These results suggest that in vivo studies of the effect of resveratrol on melanoma are warranted and that this plant polyphenol might have effectiveness as either a therapeutic or chemopreventive agent against melanoma.
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1. Introduction

Resveratrol is a polyphenol found in high concentration in red grapes, red wine, peanuts and pines [1]. In these plants resveratrol is synthesized in response to stress conditions such as an infection and thus can be considered to be a phytoalexin [2]. Resveratrol has estrogenic activity in mammals [3,4] and therefore is classified as a phytoestrogen. Resveratrol is a potent inhibitor of tumor promotion [5,6]. It has also been shown to inhibit the growth of colonic tumor cells [7], leukemic cells [8,9], breast and prostate cancer cells [10–15]. The mechanism of resveratrol action is not understood. It has antioxidant activity [16,17] and has been shown to inhibit cyclooxygenase activity [18–20].

Human melanoma is a tumor whose frequency is increasing at an alarming rate. If detected early and surgically excised the 5-year survival rate is favorable. However, later stages of the disease are difficult to treat and long-term survival is low. Interferon-γ

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* Corresponding author. Tel.: +1-304-696-7323; fax: +1-304-696-7253.
E-mail address: niles@marshall.edu (R.M. Niles).
[1] Both laboratories contributed equally to this study.

and interleukin-2 are used to treat advanced melanoma with varying degrees of success [21,22]. Thus there is a need for new agents which might have a higher therapeutic efficacy for human melanoma. In this report we demonstrate that resveratrol can inhibit the growth of both melanotic and amelanotic human melanoma cell lines. Part of this growth inhibition results from the induction of apoptosis.

2. Material and methods

2.1. Cells and culture conditions

SK-mel28 and A375 human melanoma cells were obtained from the ATCC. They were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech-Bio Inc., Kerrville, TX) and maintained in a 5% CO_2/95% air humidified atmosphere at 37 °C. Cells were grown until they were 70% confluent. They were then harvested by trypsinization (0.5% trypsin/2.6 mM EDTA), and washed with PBS. Cells were seeded into 24-well plates at a density of 2×10^4 cells/well in 1 ml of medium and allowed to attach for 24 h. The cell monolayers were washed with PBS to remove unattached cells, re-fed with fresh media with or without 30, 60, or 100 μM all-*trans*-resveratrol (Sigma Chemical, St. Louis, MO) and incubated for an additional 24–72 h. Cell number was assessed by the crystal violet assay.

2.2. Crystal violet assay

This is a colorimetric assay that is based on the uptake of crystal violet by attached cells in each well [23]. After 24, 48, and 72 h of incubation, quadruplicate wells at various concentrations of resveratrol were fixed with methanol/acetic acid (3:1) for 1 h at room temperature. The fixed cells were washed with 80% methanol and stained with crystal violet (0.5%) for 1 h. Excess dye was removed by washing the wells with distilled water. Plates were air-dried and the dye was eluted with 200 μl of 10% acetic acid. Aliquots of eluted dye from each well were transferred to 96-well plates and absorbance was measured at 595 nm with a plate reader (Microplate Bio-kinetics Reader EL312e,

Bio-Tek Instruments, Winooski, VT). Absorbance values in the resveratrol-treated cells were compared to values from cells treated with the DMSO vehicle. The data are represented graphically as the percentage of viable cells determined at various time points and resveratrol concentrations.

2.3. Annexin V assay

Perturbations in the cellular membrane occur during the early stages of apoptosis and lead to a redistribution of phosphatidylserine to the external side of the cell membrane. Annexin V selectively binds to phosphatidylserine and thus enables the use of a fluorescein-labeled annexin V kit (Trevigen, Gaithersburg, MD) to identify the cells undergoing apoptosis. Cells were also stained with propidium iodide to distinguish early apoptotic cells from necrotic cells. Ten thousand events were collected with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The percentage of live, dead and apoptotic cells was determined as discussed in the results and shown in Figs. 3 and 4. The viable cells are located in the lower left corner (negative in both annexin V-FITC and propidium iodide). Early apoptotic cells are in the lower right corner (annexin V-FITC positive). Late apoptotic cells showing signs of progressive cellular membrane and nuclear damage are in the upper right corner (double positive).

2.4. DNA laddering

Cells were incubated with 50 μM resveratrol as described above and harvested into extraction buffer (10 mM Tris–HCl (pH 7.4) containing 10 mM NaCl, 20 mM EDTA and 1% Triton X-100) after 48 h of treatment. Genomic DNA was isolated by digesting the cell extract with 10 μg/ml of proteinase K at 50 °C for 2 h. DNA was purified by phenol/chloroform, precipitated with ethanol and dissolved in TE. Integrity of the DNA was analyzed by gel electrophoresis on 1% agarose gels followed by ethidium bromide staining.

2.5. Western blotting
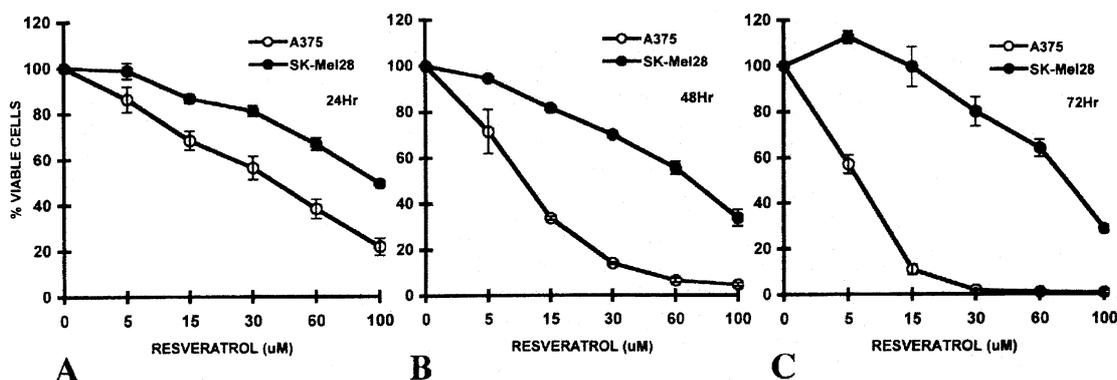
Cells were lysed in buffer A (10 mM Tris–HCl (pH

Fig. 1. Effect of resveratrol on growth of SK-mel28 and A375 melanoma. Cells were cultured for 24 (A), 48 (B) or 72 h (C) in 5–100 μM resveratrol and then stained with crystal violet as indicated in Section 2. The percentage of viable cells is calculated as (the absorbance of the resveratrol-treated cells/absorbance of the untreated cells) $\times$ 100. The values represented in the graphs are the means from two (C) or three (A,B) different experiments $\pm$ SE.

7.6), 1 mM EDTA, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin and 3.28 μg/ml PMSF) by three consecutive 10-s sonications with a Tekmar sonic disrupter at power setting 60. Proteins were separated by SDS–PAGE (5% stacking gel; 10% separating gel) and transferred to nitrocellulose (Micron Separation Inc., Westborough, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA).. The membranes were incubated with 5% non-fat dry milk overnight at room temperature. The membrane was then washed three times with TBST (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Tween-20) for 5 min each, at room temperature and then probed with an appropriate titer of antibody (Cell Signaling, Beverly, MA) that binds to either total ERK1/2, p38, JNK, or the phosphorylated (activated) version of the MAP kinases, for 1 h at room temperature. Subsequently, the membrane was washed as described above and further incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (monoclonal primary antibodies) or goat anti-rabbit IgG (polyclonal primary antibodies) (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature for 1 h. The membrane was washed three times with TBST and developed using the ECL chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). Intensity of the immunoreactive bands was quantified using a Molecular Dynamics densitometer (Sunnyvale, CA).

3. Results

Growth of the moderately pigmented, SK-mel28 and the amelanotic A375 human melanoma cell lines was differentially inhibited by resveratrol (Fig. 1A–C). A375 melanoma cells were characteristically more sensitive to resveratrol than SK-mel28. The concentration that inhibited growth by 50% in SK-mel28 cells was greater than 100 μM at 24 h of treatment (Fig. 1A), and between 60 and 70 μM at 48–72 h of treatment. In contrast, the concentration that inhibited A375 growth by 50% was 30 μM at 24 h of treatment (Fig. 1A) and between 15 and 20 μM at 48–72 h of treatment (Fig. 1B,C).

We noticed during the course of the above experiments that many cells detached from the surface of the cell culture plate, suggesting some form of cell death. To investigate this possibility, we treated SK-mel28 cells with 50 μM resveratrol for 2 days and then extracted their genomic DNA and analyzed it on agarose gels. Fig. 2 shows that DNA from control cells was intact, while DNA from cells treated with resveratrol exhibited a smear corresponding to a laddering of DNA. Attempts to show a similar DNA ladder in A375 cells treated with 50 μM resveratrol were not successful (data not shown). To further investigate these findings, we performed annexin V assays on both SK-mel28 and A375 cells treated with resveratrol. Figs. 3 and 4 show that treatment of these
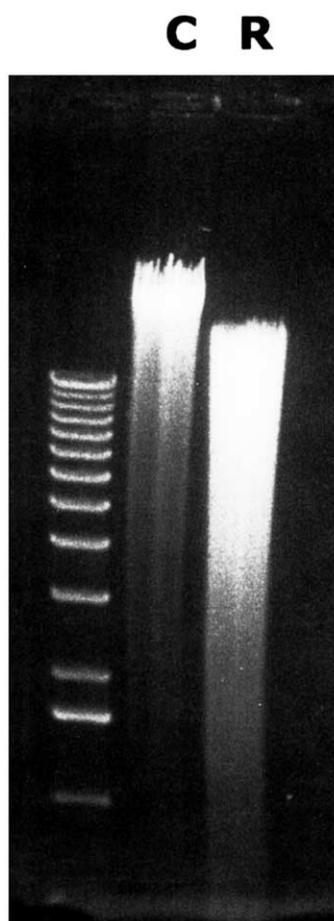
C R

Fig. 2. Effect of resveratrol on SK-mel28 human melanoma DNA integrity. SK-mel28 cells were treated with or without 30 μM resveratrol for 48 h. Genomic DNA was extracted using the 'Wizard' kit from Promega and the DNA was separated on 1% agarose gels and stained with ethidium bromide. The first lane contains a standard DNA ladder, lane two contains DNA from untreated cells and lane three contains DNA from 30 μM resveratrol-treated cells.

two human melanoma cell lines with resveratrol induces apoptosis. Early plus late apoptosis of untreated cells ranged between 1.7 and 2.5% while the percentage was 6.6% for SK-mel28 cells (Fig. 3B) and 31.1% for A375 cells (Fig. 4B) treated with 30 μM resveratrol for 48 h. We also compared the viability, via the annexin V assay, of the melanoma cell lines treated with different concentrations of resveratrol. A375 cells were considerably more sensitive to the induction of apoptosis by resveratrol

than SK-mel28 cells. Less than 10 μM resveratrol reduced viability by 50% in A375 cells, while SK-mel28 cells required 40 μM resveratrol to induce the same loss of viability.

To explore the possible pathways by which resveratrol induced apoptosis in these human melanoma cells, we determined if any of the MAP kinase pathways were activated in treated cells. This was accomplished by using antibodies against both total and phosphorylated (activated) Erk1/2, p38 and SAPK/JNK and determining the status of these proteins in control and 30 μM resveratrol-treated cells by Western blots. The results of these experiments (Fig. 5) show that Erk1/2, p38 and SAPK/JNK are expressed in both melanoma cell lines and that activated (phosphorylated) forms of SAPK/JNK, but not p38 or Erk1/2 are also present. However, treatment of A375 cells with resveratrol increased the amount of phosphorylated Erk1/2, without altering total Erk1/2 levels. Treatment of SK-mel28 cells with the same concentration of resveratrol did not result in phosphorylation of Erk1/2. Resveratrol did not alter the phosphorylation of p38 or SAPK/JNK in either cell line.

4. Discussion

In this study we have demonstrated that human melanoma cells are susceptible to resveratrol-induced inhibition of proliferation. A major portion of the decreased growth appears to be due to an induction of apoptosis. It is interesting to note the amelanotic melanoma cell line A375 was more sensitive to resveratrol-induced apoptosis than the melanotic cell line SK-Mel-28. Additional human melanoma cell lines will have to be examined in order to establish a relationship between melanin production and sensitivity to resveratrol. There appears to be only one other published report on the effect of resveratrol on melanoma. Caltagirone et al. [25] found that resveratrol inhibited the growth of the mouse melanoma B16-BL6, but did not decrease its metastatic or invasive potential.

Resveratrol has been shown to induce apoptosis in a variety of other human cancer cell lines, including breast, leukemia, prostate and colon [7–15]. In addition to inducing apoptosis, this plant compound
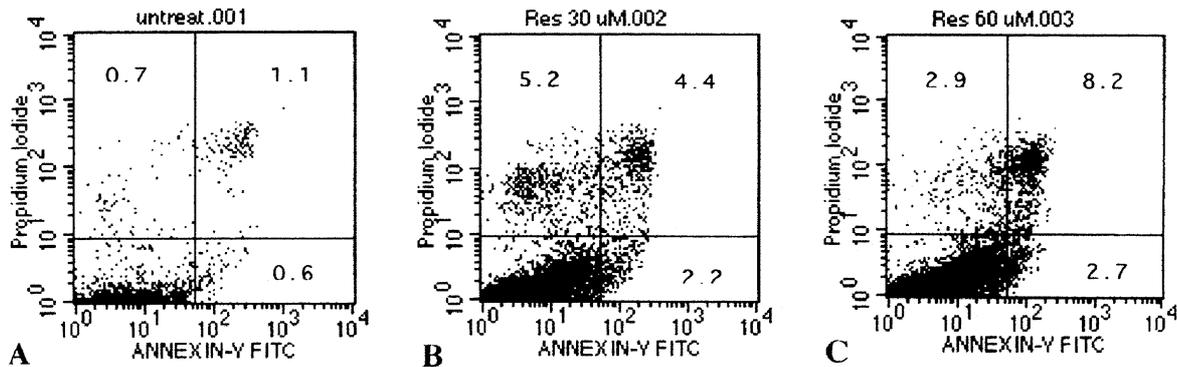
Fig. 3. Resveratrol-induced apoptosis in SK-mel28 melanoma cells. SK-mel28 cells in culture were harvested and stained with annexin V-FITC and propidium iodide (PI) according to the manufacturers' instructions. Ten thousand events were collected and analyzed for each sample (A). Cells cultured in DME with 10% FBS for 2 days. (B) Cells treated with resveratrol at 30 μM for 24 h. (C) Cells treated with resveratrol at 60 μM for 24 h. The viable cells are located in the lower left corner (negative for both annexin V-FITC and PI). Early apoptotic cells are in the lower right corner (annexin V-FITC positive). Late apoptotic cells showing signs of progressive cellular membrane and nuclear membrane damage are in the upper right corner (double positive). Necrotic cells lacking a cell membrane structure are in the upper left corner (PI positive). The numbers in the figure represent the percentage of the total cell population.

also acts as an antioxidant [17], as a phytoestrogen [3] and has been reported to inhibit lipoxygenase and cyclooxygenase activity [19]. It is not clear whether any of these activities contributes to the ability of resveratrol to induce apoptosis. The MAP kinase pathways appear to be involved in mediating apoptotic signals in a variety of cells [26–30]. Indeed we found that resveratrol increased the amount of phosphorylated Erk1/2 in A375 cells, suggesting that

this pathway was activated in these cells and may have contributed to the apoptotic pathway. Interestingly, resveratrol did not induce phosphorylation of Erk1/2 in SK-mel28 cells. Whether this difference contributes to the greater sensitivity of A375 cells to resveratrol-induced apoptosis remains to be determined. Neither p38 nor SAPK/JNK phosphorylation was altered by resveratrol treatment of either human melanoma cell line. She et al. [24] reported that
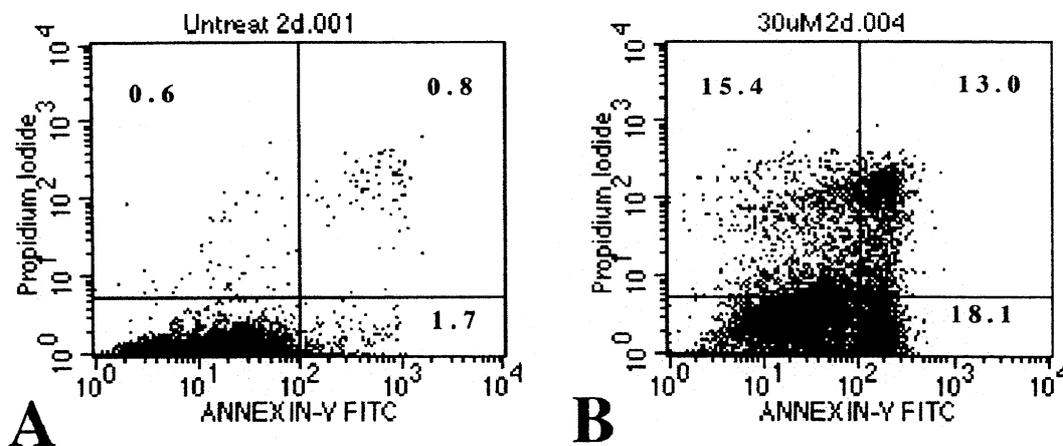

Fig. 4. Resveratrol-induced apoptosis in A375 human melanoma cells. The cells were harvested and stained with annexin V-FITC and propidium iodide (PI) according to the manufacturers' instructions. Ten thousand events were collected and analyzed for each sample. (A) Cell culture in DME with 10% FBS for 2 days. (B) Cells treated with 30 μM resveratrol for 48 h.
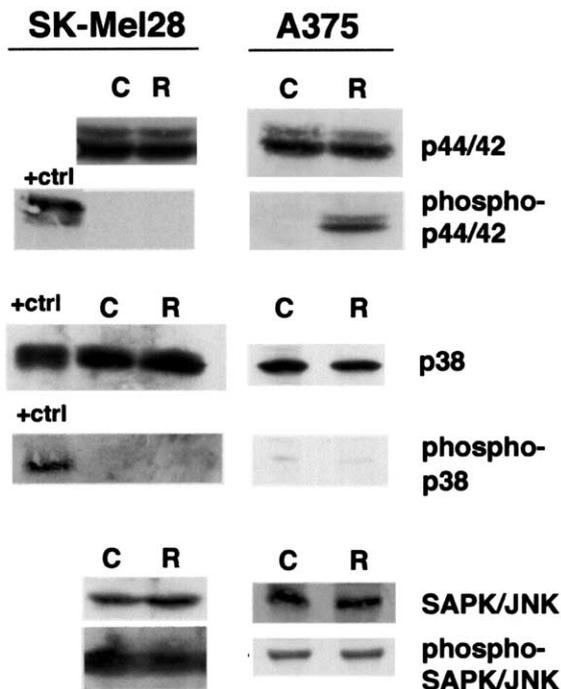
Fig. 5. Effect of resveratrol on activation of MAPK in SK-mel28 and A375 human melanoma cells. The human melanoma cells were treated with vehicle (C), or 30 μM resveratrol (R) for 24 h and then harvested and protein extracted for Western blot analysis, using total and phosphospecific antibodies to the various MAP kinases. The top panel shows total (p44/42) and phosphospecific (phospho-p44/42) ERK1/2, the middle panel shows total and phosphospecific p38 and the bottom panel shows total and phosphospecific SAPK/JNK. Positive controls (+ctrl, provided by the antibody manufacturer) demonstrate that the phosphospecific antibodies were active, despite not detecting signals, in some cases, in extracts from the melanoma cells.

resveratrol treatment of JB6 epidermal cells resulted in phosphorylation of p53 at ser 15. Mutation of ser 15 impaired the apoptotic activity of p53, suggesting that phosphorylation at this site is required for p53 to induce apoptosis [31]. In data not shown in this report, we found that neither total p53 nor phosphorylation at ser 15 was affected by resveratrol treatment of the two human melanoma cell lines. Further studies will be needed to clarify the mechanism by which resveratrol induces apoptosis in melanoma cells.

There are few effective treatments for advanced melanoma. Based on these cell culture results, studies on the ability of resveratrol to inhibit the growth of human melanoma xenografts are warranted.

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