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Inhibition of melanoma cell proliferation by resveratrol is correlated with upregulation of quinone reductase 2 and p53

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Abstract

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a grape-derived polyphenol under intensive study for its potential in cancer prevention. In the case of cultured human melanoma cells, no one to our knowledge has investigated whether resveratrol exerts similar anti-proliferative activities in cells with different metastatic potential. Therefore, we examined the effects of this polyphenol on the growth of weakly metastatic Line IV clone 3 and on autologous, highly metastatic Line IV clone 1 cultured melanoma cells. Comparable inhibition of growth and colony formation resulted from treatment by resveratrol in both cell lines. Flow cytometric analysis revealed that resveratrol-treated clone 1 cells had a dose-dependent increase in S phase and a concomitant reduction in the G_1 phase. No detectable change in cell cycle phase distribution was found in similarly treated clone 3 cells. Western blots demonstrated a significant increase in the expression of the tumor suppressor gene p53, without a commensurate change in p21 and several other cell cycle regulatory proteins in both cell types. Chromatography of Line IV clone 3 and clone 1 cell extracts on resveratrol affinity columns revealed that the basal expression of dihydronicotinamide riboside quinone reductase 2 (NQO2) was higher in Line IV clone 1 than clone 3 cells. Levels of NQO2 but not its structural analog NQO1 were dose-dependently increased by resveratrol in both cell lines. We propose that induction of NQO2 may relate to the observed increased expression of p53 that, in turn, contributes to the observed suppression of cell growth in both melanoma cell lines.

Keywords: Resveratrol; Resveratrol targeting protein NQO2; Immobilized resveratrol affinity columns; Melanoma carcinogenesis

Cancer presents clinically as a constellation of diseases and at all stages. From primary prevention to therapeutic management and treatment, nutrition is a key factor. Decades of epidemiological studies have established a direct link between consumption of plant-based diets and a reduction in risk of cancer [1–4]. In recent years, efforts directed at the identification of bioactive ingredients in those diets and delineation of their mechanism(s) have demonstrated that even ubiquitous, non-nutritional secondary plant metabolites, such as flavonoids and polyphenolics widely present in foods consumed in the US, have significant health consequences [5–10].

Resveratrol is a phytoalexin found in grapes, nuts, and red wine. Various studies have reported that resveratrol has antioxidant, antithrombotic, anti-inflammatory, anti-aging, cardioprotective, and anti-tumorigenic properties [11–18]. We had previously proposed that part of its diverse biological attributes might relate to the ability of resveratrol to interact with specific cellular proteins, denoted resveratrol targeting proteins (RTPs) [19]. To test this hypothesis, we designed an affinity matrix with resveratrol immobilized on epoxy-activated agarose beads that served as a selection platform for the isolation and identification of RTPs. The efficacy of this approach was recently demonstrated

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by isolating a 22-kDa polypeptide, designated RTP-22, from prostate and erythroleukemia K562 cancer cells that was identified as dihydronicotinamide riboside quinone reductase 2 (NQO2) by MALDI-TOF mass spectrometry and cloning [19,20].

Melanoma is the most common fatal cutaneous malignancy in Caucasian populations. The frequency of melanoma has increased by a factor of 15 over the past 60 years and it is continuing to rise at an annual incidence rate of 5–7% in fair-skinned individuals despite advances made in skin cancer detection and diagnosis [21–23]. The prognosis for individuals with advanced cutaneous malignant melanoma is dismal due to the lack of effective treatment options [24–26].

Chemoprevention has recently been proposed as a new approach in the control of melanoma [27,28]. In the case of cultured human melanoma cells, no one to our knowledge has investigated whether resveratrol exerts similar anti-proliferative activities in cells with different metastatic potential. Therefore, we tested the effects of resveratrol on the growth of highly metastatic Line IV clone 1 cultured melanoma cells and on autologous, weakly metastatic Line IV clone 3 cells derived from the same donor. These two cell lines have previously been used in a model system to identify differentially expressed cellular antigens associated with the metastatic phenotype [29,30]. We found that treatment by resveratrol significantly inhibited the proliferation of both cell types. The suppression of proliferation in Line IV clone 1 cells was correlated with a threefold increase in the S and a corresponding 75% reduction in G_1 phases of the cell cycle. However, reduction in growth of Line IV clone 3 was not associated with changes in cell cycle events. Immunoblot analysis showed that NQO2 expression was significantly higher in Line IV clone 1 than clone 3 cells, and that its levels were dose-dependently increased by resveratrol to a greater degree in clone 1 than clone 3. The increase in NQO2 was correlated with upregulation of expression of tumor suppressor gene protein p53. We propose that the induction of NQO2 by resveratrol may be coupled to an increase in stability of p53 that, in turn, contributes to the observed suppression of cell growth in both human melanoma cell lines.

Materials and methods

Materials. Resveratrol was purchased from LKT Laboratories (St. Paul, MN) or Sigma Chemical (St. Louis, MO). Epoxy-activated agarose resin (12 atom linker, 33 μmol of epoxy group per ml of packed gel) was also obtained from Sigma Chemical. Other biochemical and molecular biology grade reagents were purchased from various commercial vendors. Stock solutions of resveratrol (12.5 mM) were prepared in dimethyl sulfoxide (DMSO) and kept at −20 °C.

Cell cultures. Autologous human melanoma Line IV clone 1 and clone 3 cells were sub-clones of parental Line IV cells, originally established from a primary malignant melanoma lesion [29]. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. Cells were split once a week and media were changed every 3–4 days.

Cell proliferation and colony formation assays. Line IV clone 1 and clone 3 cells were seeded at 5×10^4 cells/ml in T-75 flasks, allowed to attach overnight, and treated for 72 h with various doses of resveratrol. Control and resveratrol-treated cells were harvested by trypsinization and cell numbers were determined using a hemocytometer. Cell viability was assayed by trypan blue dye exclusion [31–34].

Colony formation or clonogenicity was performed as described [33,34]. Two milliliter aliquots of Line IV clone 1 and clone 3 cells adjusted to a density of 200 cells/ml using RPMI 1640 containing 10% FBS were dispensed into individual wells of six-well tissue culture dishes, followed by addition of different concentrations of resveratrol or 0.2% DMSO. Following another 14 days in culture, the cells were fixed and stained with 0.1% crystal violet to visualize colonies.

Flow cytometry. Line IV clone 1 and clone 3 cultures were treated with increasing concentrations of resveratrol for 3 days and harvested. Cells were washed once with PBS and stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Triton X-100 (Sigma) at pH 6.8 as previously described [31,32,34,35]. The DNA-specific DAPI fluorescence was excited with UV light and collected with appropriate filters in an ICP-22 (Ortho Diagnostic, Westwood, MA) flow cytometer. The cell cycle distribution was obtained by deconvoluting the DNA content frequency histograms with the use of Multicycle software (Phoenix Flow, San Diego, CA).

Preparation of immobilized resveratrol affinity column. A chromatographic matrix with resveratrol as the immobilized affinity ligand was prepared as described [19]. In brief, resuspended epoxy-activated agarose was reacted with resveratrol dissolved in 0.1 M NaOH. Chemical coupling of ligand to the solid support was terminated by addition of 1 M sodium acetate buffer (pH 5.0) containing 1 mM dithiothreitol (DTT). The affinity resin containing immobilized resveratrol was stored in 0.1 M sodium acetate, pH 5.0, containing 1 mM DTT. Control resins consisted of mock-treated beads (identical procedure except that no resveratrol was added) or beads immobilized with tyrosine as the ligand.

Fractionation of cytoplasmic extracts on immobilized resveratrol affinity columns. Cytoplasmic extracts from cultured melanoma cells were prepared as described previously [19,31,35]. In brief, cells were suspended in buffer containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM DTT, 0.5% NP-40, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μl/ml of the protease inhibitor cocktail from Sigma Chemical. Cells were lysed by vortexing in three freeze–thaw cycles and cell-free extracts were obtained by a 10-min centrifugation in a refrigerated microcentrifuge. The protein concentration of cytoplasmic extracts was determined using a protein assay kit, purchased from Pierce Chemical. All samples were stored in aliquots at −80 °C.

To characterize proteins bound to the resveratrol affinity column, extracts containing 0.6–1.0 mg of protein in 200 μl of lysis buffer were individually mixed with 50 μl control (mock-treated or tyrosine-linked) or resveratrol immobilized agarose beads in a 1.5-ml Eppendorf tube. The tube was incubated overnight at 4 °C with mild tumbling. The gel slurry containing protein extract was loaded onto a minicolumn (from Pierce Chemical) and washed with 10–20 ml of lysis buffer to remove unbound proteins. The column was next eluted five times, each time with 0.5 ml lysis buffer containing 0.35 M NaCl, and was followed by the same number of washings using 1 M NaCl supplemented buffer. Next, the column was re-equilibrated with the lysis buffer, and eluted with 1 mM ATP. The last step involved elution with 1–2 mM resveratrol dissolved in 2% DMSO. Specificity of binding was demonstrated by mixing extracts with 1 mM resveratrol prior to binding to the affinity resin, for ascertaining competition of binding of distinct RTPs. Proteins eluted by salt, 1 mM ATP, and finally resveratrol in the last

step were analyzed by 10% SDS–PAGE and visualized by silver staining.

Western blot analysis. To analyze the expression of specific proteins in control and treated cell extracts, 10 μg proteins was boiled for 5 min in Laemmli buffer and separated on 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were then transferred to nitrocellulose membranes by a semi-dry transfer method. After blocking with TBST containing 5% low-fat milk, the membranes were probed for the level of expression of cyclin D1, Rb, PCNA, p53, p21, quinone reductase types 1 and 2 (NQO1 and NQO2), and β-actin using monoclonal or polyclonal antibodies, as described [31–34]. Specific immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) or color reaction using procedures detailed in the manufacturer's protocol (Kirkegared & Perry Laboratories, Gaithersburg, MD).

Activity assay of dihydronicotinamide riboside (NRH):quinone reductase 2, NQO2. NQO2 was assayed as NRH-dependent reduction of menadione, assayed spectrophotometrically as the coupled reduction of MTT at 610 nm at 25 °C in the presence of dicoumarol (inhibitor of NQO1). The assay mixture contained 100 mM Tris–HCl, pH 8.5, 10 μM menadione, 160 μM NRH, 0.3 mg/ml MTT, and 10 μM dicoumarol.

Results

Suppression of growth and clonogenicity of Line IV clone 1 and IV clone 3 human melanoma cells by resveratrol

We first tested the anti-proliferative effects of resveratrol on in vitro growth of Line IV clones 1 and 3 cells by adding varying concentrations of resveratrol to tissue culture media and following the growth of these cells. At various times, control and treated cells were harvested by trypsinization. Cell viability was determined by incubation with trypan blue and the numbers of blue (dead) cells and transparent (live) cells were counted in a hemocytometer. Fig. 1 depicts a 72-h treatment experiment that demonstrated suppression of proliferation by resveratrol in both Line IV clone 1 and Line IV clone 3 cells. The Line IV clone 1 cells appeared to be slightly more sensitive to the grape-derived polyphenol than

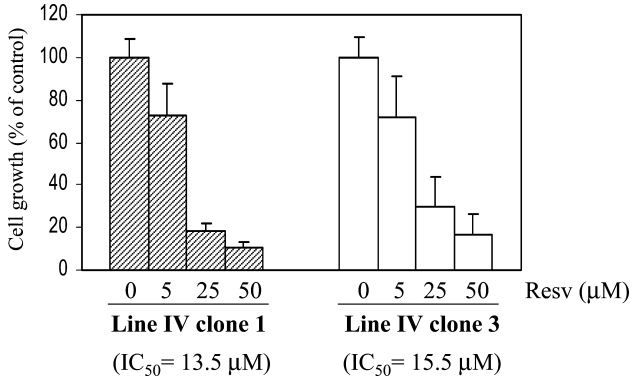


Fig. 1. Effects of resveratrol on growth of Line IV clone 1 and Line IV clone 3 human melanoma cells. Growth of Line IV clone 1 and clone 3 cells is inhibited by adding varying doses of resveratrol as described under Materials and methods. Results represent the average of 2–3 experiments.

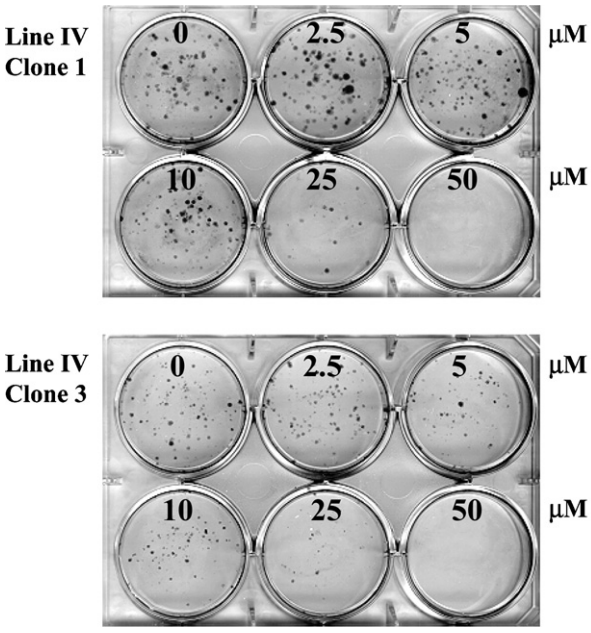


Fig. 2. Effects of resveratrol on clonogenicity of human melanoma Line IV clone 1 and clone 3 cells. The clonogenicity of Line IV clone 1 and clone 3 cells is reduced in a dose-dependent manner by resveratrol treatment. The clonal growth assay was performed as described under Materials and methods. Colony formation was visualized by fixing and staining the cells with 0.1% crystal violet. Experiments were performed in duplicate or triplicate.

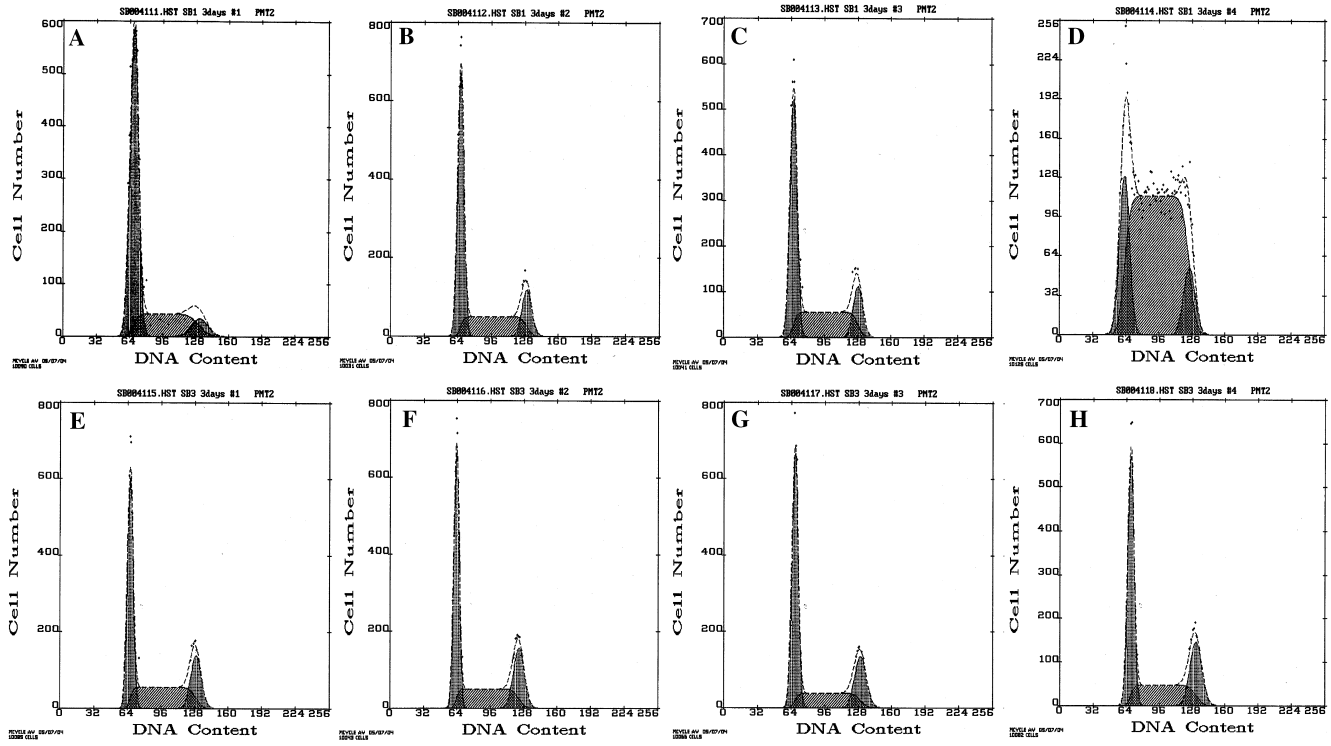
Line IV clone 3 cells. Additional evidence for the growth inhibitory activity of resveratrol on human melanoma cells was based on assessment of the ability of tumor cells to grow and form foci, i.e., an indirect measurement of the propensity of tumor cells for neoplastic transformation. This assay is known as colony formation or clonogenicity and is typically performed by plating a fixed number of Line IV clone 1 and clone 3 cells onto multi-well tissue culture dishes, with and without addition of various doses of resveratrol. Colony formation after 14 days in culture can be visually inspected by fixing and staining the cells in 0.1% crystal violet. Fig. 2 shows that clonogenicity of both Line IV clone 1 and clone 3 melanoma cell lines was significantly inhibited by resveratrol, as evident by a marked reduction in both the number and the size of colonies at $\geqslant 10$ μM resveratrol. Notably, Line IV clone 1 cells formed larger and greater number of colonies, possibly a reflection of their higher metastatic potential as compared to Line IV clone 3 cells.

Effects of resveratrol on cell cycle progression and induction of apoptosis in melanoma cells

To explore the underlying mechanism for the observed anti-proliferative effects of resveratrol, cell cycle analyses were performed. Cell cycle phase distribution in Line IV clone 1 and clone 3 cells, with and without

treatment with 5, 25, and 50 μM resveratrol, is depicted in Fig. 3. Resveratrol had little effect on cell cycle transition in Line IV clone 3 cells, whereas it dose-dependently suppressed progression of clone 1 cells. This effect was most vividly illustrated in Line IV clone 1 cells treated with 50 μM resveratrol; the proportion of S phase cells increased almost threefold from 28.6% to 75.4%, and G_1 cells decreased by 75% from 64% to 16.6% (Fig. 3). Interestingly, although resveratrol elicited such a pronounced derangement in cell cycling, it had little to no effect on induction of apoptosis, recognized as the presence of cells with fractional DNA content, in either cell type.

To gain additional information on the cell cycle effects of resveratrol in Line IV clone 1 cells, changes in cell cycle regulatory protein levels were monitored by immunoblot analysis. Representative results in Rb and p53 expression are shown in Fig. 4. The retinoblastoma tumor suppressor protein Rb, whose expression and state of phosphorylation play a pivotal role in the control of cell cycle checkpoints, was only slightly reduced by treatment with 50 μM resveratrol in both clone 1 and clone 3 cells. Although resveratrol did not affect cell cycling in clone 3 cells, it caused a more pronounced decrease in hyperphosphorylated Rb. The most dramatic effect of resveratrol was a dose-dependent increase in the expression of the tumor suppressor gene p53. Indeed, in the case of Line IV clone 1 cells, as little as 5 μM resveratrol sufficed to upregulate p53 (Fig. 4). Since p53 functions as a transcriptional activator of a number of genes, including the checkpoint regulator p21, we tested whether resveratrol correspondingly



Treatment	Line IV clone 1				Line IV clone 3			
	A	B	C	D	E	F	G	H
	Control	5 μM	25 μM	50 μM	control	5 μM	25 μM	50 μM
G1	64.1	52.9	48.4	16.6	45.0	46.3	53.8	46.6
S	28.6	33.0	37.6	75.4	36.6	32.0	25.9	31.1
G2/M	7.3	14.1	14.1	8.0	18.4	21.8	20.3	22.3

Fig. 3. Effects of resveratrol on changes in cell cycle phase distribution. (A–D) Cell cycle phase distribution of Line IV clone 1 cells and (E–H) results from Line IV clone 3 cells. Cells were treated for 72 h with 0, 5, 25, and 50 μM resveratrol. Results of the cell cytometric analysis appear in the table.

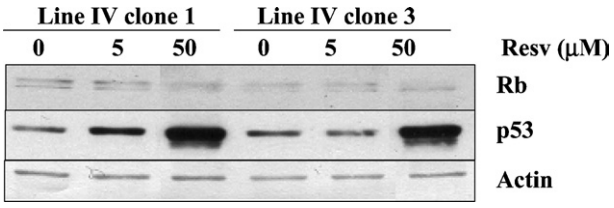


Fig. 4. Expression of Rb, p53, and actin in human melanoma Line IV clone 1 and Line IV clone 3 cells. Western blot analysis of extracts prepared from cells treated for 3 days with 0, 5, 25, and 50 μM resveratrol.

affected p21. Contrary to expectation, p21 levels were reduced by $\geqslant 5$ μM resveratrol in Line IV clone 1, whereas in Line IV clone 3 cells only 50 μM resveratrol had an appreciable suppressive effect on p21 (data not shown).

Identification of cellular proteins distinguishing Line IV clone 1 and clone 3 cells and their differential responses to resveratrol by resveratrol affinity chromatography

To determine differences between Line IV clone 1 and clone 3 cells, and analyze and identify targets that might mediate the growth sensitivity and responsiveness of these two human melanoma cells to resveratrol, an affinity chromatography approach was used. Resveratrol was immobilized on epoxy-activated agarose beads, thereby providing an affinity platform to fractionate control and treated cell extracts. This resveratrol-directed fractionation strategy relies on the molecular prowess (specificity and affinity) of resveratrol and not a priori assumption of relevant cellular attributes of the melanoma cells in question; as such, it could generate protein profiles characteristic of the cell type being analyzed. Increasing salt concentrations and ATP were used to elute the column in order to reduce binding of nonspecific proteins to the affinity column. Cellular targets with distinct binding affinity to resveratrol can be displaced from the column using resveratrol. Results comparing fractionation of extracts prepared from untreated Line IV clone 1 and clone 3 cells are depicted in Fig. 5. Visual inspection of the silver-stained protein bands corresponding to the various elution conditions revealed the following: (1) major differences can be observed with respect to proteins eluted with 1 M NaCl, 1 mM ATP, and also resveratrol; (2) a protein, identified as RTP-22, was found in the resveratrol-eluted fraction and was present proportionately much more abundant in Line IV clone 1 than Line IV clone 3 extracts; (3) RTP-22 is identified as a resveratrol binding protein known as dihydronicotinamide riboside quinone reductase 2 (NQO2), on the basis of its migration in SDS–PAGE, its competition from binding to the resveratrol affinity column by adding excess resveratrol to the extract prior to fractionation (compare lanes 4 with 5 for Line IV clone 1 and lanes 8 with 9 for Line IV clone

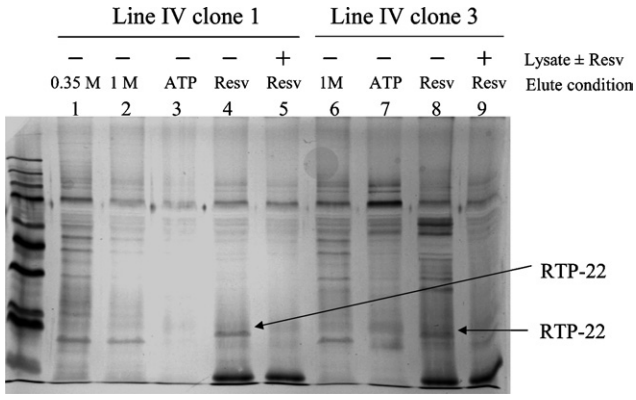


Fig. 5. Analysis of melanoma proteins on resveratrol immobilized affinity columns. Lysates from Line IV clone 1 and clone 3 cells were fractionated on resveratrol affinity columns, as detailed under Materials and methods. Fractions eluted with 0.35 M and 1 M NaCl, followed by elution using 1 mM ATP, and in the last step elution with 1–2 mM resveratrol were concentrated, separated by SDS–PAGE, and visualized by silver-staining. Samples of both Line IV clone 1 and clone 3 cell extracts, containing 1 mM resveratrol, were prepared prior to binding and fractionation on the resveratrol affinity column as specificity controls for protein binding to resveratrol.

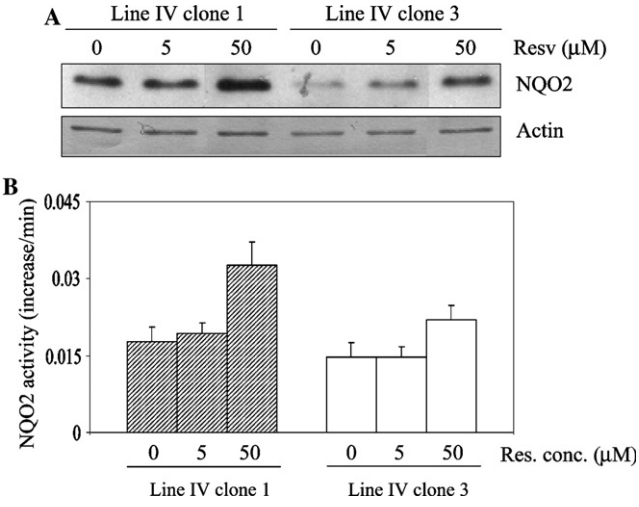


Fig. 6. Induction of NQO2 by resveratrol in human melanoma Line IV clone 1 and clone 3 cells. (A) Western blot analysis to determine the expression of NQO2 in Line IV clone 1 and clone 3 cells treated for 3 days with 0, 5, and 50 μM resveratrol. (B) Activity assay of NQO2 as detailed under Materials and methods.

3), NQO2 activity assays, and by Western blot analysis (Fig. 6).

Induction of NQO2 by resveratrol in Line IV clone 1 and clone 3 cells

Using activity assays and Western blot analysis, we also checked for effects of resveratrol on expression of NQO2 in both cell lines. These experiments showed that the levels of NQO2 were increased by resveratrol in a dose-dependent manner, and that the magnitude of

induction was slightly more pronounced in Line IV clone 1 than Line IV clone 3 cells (Fig. 6). Treatment with resveratrol had no appreciable effect on the expression of NQO1, a structural analog of NQO1, in these two human melanoma cell lines (data not shown). These results as a whole demonstrate qualitative and quantitative differences between Line IV clone 1 and clone 3 human melanoma cells. Since the magnitude of change in NQO2, in response to resveratrol, correlated well with the observed increase in p53, we propose that NQO2 levels may be coupled to elevated expression of p53 that, in turn, contributes to observed suppression of cell growth in both melanoma cell lines.

Discussion

We have demonstrated in the present studies that resveratrol is a potent inhibitor of proliferation and of colony formation in highly metastatic Line IV clone 1 and weakly metastatic Line IV clone 3 human melanoma cells. Notably, these two cell lines were sub-clones of the original Line IV cells established from a single primary melanoma lesion [29,30] whose qualitative differences in metastasis have been demonstrated by subdermal challenge in nude mice. Suppression of proliferation in these two melanoma cells by resveratrol was significantly more pronounced and occurred at a lower dose than what had been previously reported in prostate and breast cancer cells [32,36–38], and also in SK-mel28 melanoma cells [39] although comparable to that found in A375 melanoma cells [39,40]. Therefore, this grape-derived polyphenol should be further explored for its potential in the prevention of human melanoma.

Suppression of cell proliferation in resveratrol-treated cells was accompanied by an accumulation in S phase in Line IV clone 1 but not in clone 3 cells, which was most evident in cells treated with 50 μM resveratrol (Fig. 3). This could be explained either by their arrest in S, increased transit time through this phase, or apoptosis in G_1 and G_2/M phases of the cell cycle. Since resveratrol had no demonstrated effect on the induction of apoptosis, based on flow cytometric analysis, in either Line IV clone 1 or clone 3 cells, it seems an unlikely explanation for the observed changes in cell cycle distribution. Western blot analysis of control and treated cell extracts showed a reduction in hyperphosphorylated and unphosphorylated forms of Rb, more substantially in 50 μM resveratrol-treated Line IV clone 1 than clone 3 cells. Similarly, a striking increase was also seen in the expression of tumor suppressor gene p53, without a corresponding change in its downstream target p21 (Fig. 4). These results could contribute to the observed suppression in cell growth in both cell types, albeit by mechanisms unrelated to p53-dependent upregulation of p21 expression. It is notable that resveratrol has been reported to inhibit ribonucleotide reductase and DNA polymerase δ [41,42], both enzymes that affect DNA replication, these effects could provide a mechanistic basis for the observed prolongation of resveratrol-treated cells in S phase and is an area of current research focus.

To obtain additional insights on cellular targets that might mediate the anti-proliferative mechanism of resveratrol, we tested the efficacy of resveratrol-tagged affinity columns to microcapture RTPs from weakly metastatic Line IV clone 3 cell extracts, for comparison with protein patterns generated from highly metastatic Line IV clone 1 cells. This is a strategy that permits probing of low abundance proteins and could generate protein profiles serving to distinguish normal from tumor cells and tumor cells with different metastatic potentials. It is noteworthy that this experimental approach has led to the capture and cloning of NQO2 in human PC-3 prostate cancer and K562 erythroleukemia cells [19,20]. Chromatography of extracts from Line IV clone 3 and clone 1 cells on resveratrol-immobilized affinity columns shows that NQO2 is present at a higher level in clone 1 than clone 3 cells (Figs. 5 and 6). Treatment of both cell types by resveratrol resulted in substantial induction of NQO2, as verified by activity assays and immunoblot analysis with a NQO2-specific polyclonal antibody (Fig. 6). Since resveratrol treatment did not induce changes in NQO1 (data not shown), a structural analog of NQO2, and because increased expression of NQO2 is highly correlated with induction of p53 by resveratrol in both melanoma cell lines, we hypothesize that NQO2 plays a critical role in the suppression of melanoma cell growth by resveratrol. The potential of NQO2 to bind to and stabilize p53 has been previously suggested by Jaiswal and coworkers [43].

In summary, resveratrol significantly reduces cell proliferation in highly metastatic Line IV clone 1 cells, probably by impairing the progression of cells through the S into G_2M phases of the cell cycle. Moreover, the growth suppressive effect of resveratrol was similarly found in the weakly metastatic Line IV clone 3 cells, by as yet undetermined mechanisms. Chromatography of both melanoma cell extracts on resveratrol-immobilized columns shows that there are quantitative and qualitative differences in the profile of proteins RTPs retained on this affinity platform. The identity of one RTP has been determined as NQO2, which shows a higher expression in Line IV clone 1 than Line IV clone 3 cells. Expression of NQO2 was copiously induced by resveratrol in both cell types. The identity and functions of other RTPs in both melanoma cell types remain to be elucidated. Conceivably, their identification and characterization may be of fundamental importance in the biology of melanoma, particularly in the context of metastasis and in regard to chemoprevention of melano-

ma by resveratrol. Since foods rich in resveratrol, such as grapes and peanuts, are popular items of the American diet, it is possible that resveratrol may be considered as a chemopreventive agent for patients at risk of recurrent melanoma.

Acknowledgments

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