

Resveratrol Enhances Radiosensitivity of Human Non-Small Cell Lung Cancer NCI-H838 Cells Accompanied by Inhibition of Nuclear Factor-Kappa B Activation

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Resveratrol/NF- κ B/Radiation/Lung cancer.

Resveratrol, a polyphenol in red wine, possesses many pharmacological activities including cardio-protection, chemoprevention, anti-tumor effects, and nuclear factor-kappa B (NF- κ B) inactivation. The present study was designed to evaluate the effects and possible mechanism of resveratrol in enhancing radiosensitivity of lung cancer cells. Human non-small cell lung cancer NCI-H838 cells were irradiated with or without resveratrol pretreatment. The surviving fraction and sensitizer enhancement ratio (SER) were estimated by using a colony formation assay and linear-quadratic model. The cell-cycle distribution was evaluated by using propidium iodide staining and flow cytometry. An ELISA-based assay with immobilized oligonucleotide was performed to assess the DNA binding activity of NF- κ B. Resveratrol had no direct growth-inhibitory effect on NCI-H838 cells treated for 24 hours with doses up to 25 μ M. Pretreatment with resveratrol significantly enhanced cell killing by radiation, with an SER up to 2.2. Radiation activated NF- κ B, an effect reversed by resveratrol pretreatment. Resveratrol resulted in a decrease of cells in the G₀/G₁ phase and an increase in the S phase. Our results demonstrate that resveratrol enhances the radiosensitivity of NCI-H838 cells accompanied by NF- κ B inhibition and S-phase arrest.

INTRODUCTION

The incidence of locally advanced lung cancer is increasing, but we still don't have reliable treatment. Concurrent use of radiotherapy and chemotherapy (such as cisplatin, etoposide, gemcitabine, taxanes, etc.) has been used to control locally advanced non-small cell lung cancer. Although the therapeutic efficacy has been moderately improved, a marked increase in adverse effects remains to be resolved. Therefore, it is important to develop novel drugs, which may sensitize cancer cells to radiation while protecting normal tissue.

A possible agent for this purpose is resveratrol (trans-3,4',5-trihydroxystilbene), a polyphenol found in grape skin. Epidemiological studies have indicated that the consumption of red wine reduces mortality from coronary heart disease.^{1,2)} The cardioprotective effect has been attributed to the polyphenol fraction of red wine, a key component of which is resveratrol. Red wines contain varying concentration of resveratrol, from 1 to 75 mg/liter.³⁾ Resveratrol is found in other various fruits and vegetables as well and functions as a phytoalexin that protects against fungal infections.⁴⁾

Resveratrol has been shown to inhibit the growth of a wide variety of malignant cells, including leukemia, prostate, and breast cancer cells.^{5–7)} Its ability to induce expression of FasL, p53, and p21 may contribute to its growth-inhibitory effects.⁶⁾ The chemopreventive effect of resveratrol may be mediated by suppression of cyclooxygenase-2 (COX-2), cytochrome p450, and c-fos expression.^{8–10)}

Resveratrol has been reported to block TNF-induced activation of NF- κ B in U937, Jurkat, HeLa and H4 cells.¹¹⁾ Adhami et al reported that resveratrol blocked ultraviolet B-mediated activation of NF- κ B in normal human epidermal keratinocytes¹²⁾ and prevented ultraviolet B-induced damage via inhibition of COX and ornithine decarboxylase enzyme activities.¹³⁾ It was found to enhance radiation-induced apoptosis in human cancer cell lines, including HeLa (cervical

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carcinoma), K-562 (chronic myeloid leukemia) and IM-9 (multiple myeloma) cells.¹⁴ The radiosensitizing effect on HeLa and SiHa cells was accompanied by early S-phase cell cycle checkpoint arrest.¹⁵ Fiore *et al* demonstrated that combination of resveratrol and X ray, in comparison with X ray alone, increased the DNA breaks and blocked the cell cycling at early S phase.¹⁶

NF- κ B activation induced by radiation may be involved in the radio-response of several tumor cells.^{17,18} Resveratrol has been reported to inhibit NF- κ B in human tumor cells, including lung cancer A549 cells.¹⁹ We hypothesized that the NF- κ B-inhibiting effect of resveratrol may be the means by which it augments radiation-induced cytotoxicity. The goal of this study was to investigate if resveratrol pretreatment sensitizes human non-small cell lung cancer NCI-H838 cells to radiation and to explore the possible mechanisms of such an effect.

MATERIALS AND METHODS

Cell cultures

The human NSCLC cell line NCI-H838, originally derived from a primary adenocarcinoma of the lung in a 59-year-old Caucasian man, was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in a culture using DMEM with 100 units/mL penicillin, 100 μ g/mL streptomycin, 2 mM-glutamine (all from Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) at 37°C in a humidified 5% CO₂ atmosphere.

Chemicals and reagents

Resveratrol (trans-3,4,5-trihydroxystilbene, structure demonstrated in Fig. 1) was purchased from Sigma (St. Louis, MO). Confluent cultures of cells were exposed to resveratrol dissolved in dimethyl sulfoxide (DMSO). Control cells were exposed to DMSO only. The final concentration of DMSO was adjusted to less than 0.1% (v/v).

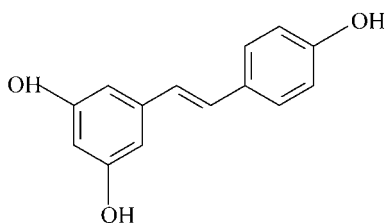


Fig. 1. Chemical structure of resveratrol.

Cell viability assessment

To determine the effect on cell viability, NCI-H838 cells were treated with various doses (0, 6.25, 12.5, 25, 50 μ M) of resveratrol for 24 h. Cell viability was assessed by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay.

Resveratrol treatment and radiation delivery

For radiation sensitization, cells were pretreated with various doses (as noted above) of resveratrol for 24 h. After the cells were washed, 6 MeV of electron beam energy was delivered by a linear accelerator (Clinac[®] 1800, Varian Associates, Inc., CA. dose rate 2.4 Gy/min) with various doses (0.5, 1, 2 and 3 Gy) in a single fraction. Full electron equilibrium was ensured for each fraction by a parallel plate PR-60C ionization chamber (CAPINTEL, Inc., Ramsey, NJ). Following radiation, cells were plated for a colony formation assay. In some experiments, resveratrol was not washed out to see the effect of different timing of resveratrol treatment.

Colony formation assay

Viable tumor cells (10^3) were plated into each 35-mm culture dish and allowed to grow in McCoy's 5A medium containing 20% heat-inactivated FCS and 0.24% agarose at 37°C in a humidified 5% CO₂ incubator. After 10 to 14 days, the dishes were stained with 0.4% crystal violet and colonies containing ≥ 50 cells were counted. The surviving fraction was calculated as mean colonies/(cells inoculated \times plating efficiency). The control plating efficiency for NCI-H838 cells was 57%. Survival curves were fitted by a linear-quadratic model.²⁰ The sensitizer enhancement ratio (SER) was calculated as the radiation dose needed for radiation alone divided by the dose needed for resveratrol plus radiation to yield a surviving fraction of 37%.

Cell cycle analysis by flow cytometry

After 24 hours' treatment with 25 μ M resveratrol, cells were harvested and fixed with 70% ethanol at 4°C for 1 h. The cells were stained for 30 min with propidium iodide solution (propidium iodide, 0.5 mg/mL; RNase, 0.1 mg/mL) contained in a CycleTEST PLUS DNA reagent kit (Becton Dickinson, Lincoln Park, NJ). Analysis of the DNA content was performed using a FACScaliber flow cytometer (Becton Dickinson). The data from 10^4 cells were collected and analyzed using ModFit software (Becton Dickinson). To determine whether the effect of resveratrol on cell cycle modulation is reversible or not, H838 cells treated for 24 h with resveratrol 25 μ M were washed and fed with fresh medium. After serial releasing times (4, 8 and 24 h), the cell cycle distribution was examined.

ELISA-based assay for DNA binding activity of NF- κ B

The DNA binding activity of NF- κ B was assessed using a Trans-AM ELISA-based kit purchased from Active Motif (Carlsbad, CA), following the manufacturer's instructions. Briefly, the NF- κ B consensus sequence (5'-GGGACTTTC-3') was already immobilized on a 96-well plate. Nuclear extracts were added to the plate and incubated for 1 h at room temperature. Activated NF- κ B from nuclear extracts specifically bound to the immobilized oligonucleotide was detected using antibody to NF- κ B p65 followed by a horse-radish per-

oxidase-conjugated secondary antibody. We assessed p65 because it is a major NF- κ B subunit that accumulates in the nucleus after NF- κ B activation. The development of the reaction complex was determined by the absorbance at 450 nm wavelength. This method is an alternative to the gel shift assay for assessing NF- κ B activity quantitatively.^{21,22)}

Assay for nuclei fragmentation

After seeding for 24 h, NCI-H838 cells were treated with vehicle, resveratrol 25 μ M for 24 h, radiation 2 Gy, resver-

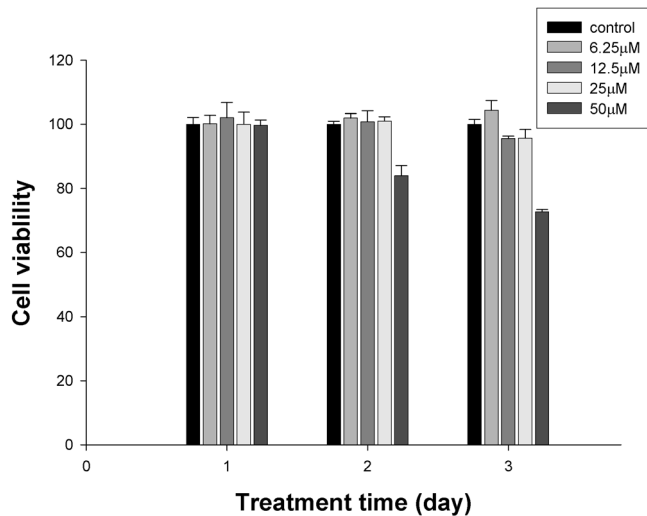


Fig. 2. The effect of resveratrol on cell viability of NCI-H838 cells. Cells were treated with resveratrol (0 to 50 μ M) for 24 h and were then collected and subjected to MTT assay. Data from three separate experiments are presented as mean \pm SEM.

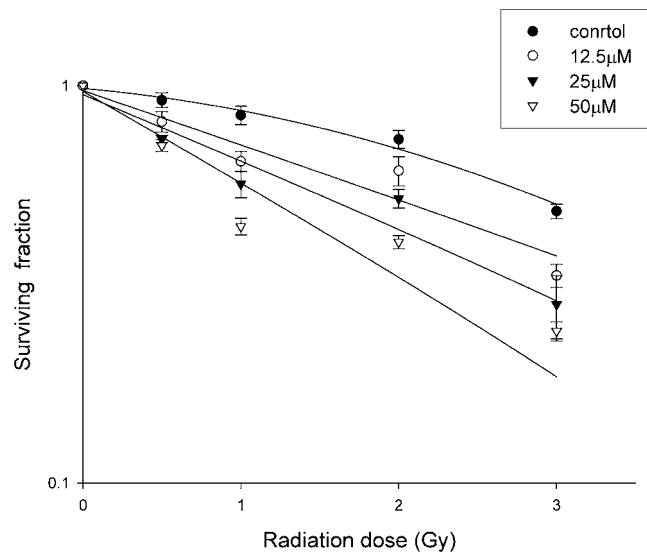


Fig. 3. Radiation survival curves for NCI-H838 cells. Cells were treated with vehicle (●), resveratrol 12.5 μ M (○), 25 μ M (▼), or 50 μ M (▽) for 24 h before radiation. Data from three separate experiments are presented as mean \pm SEM.

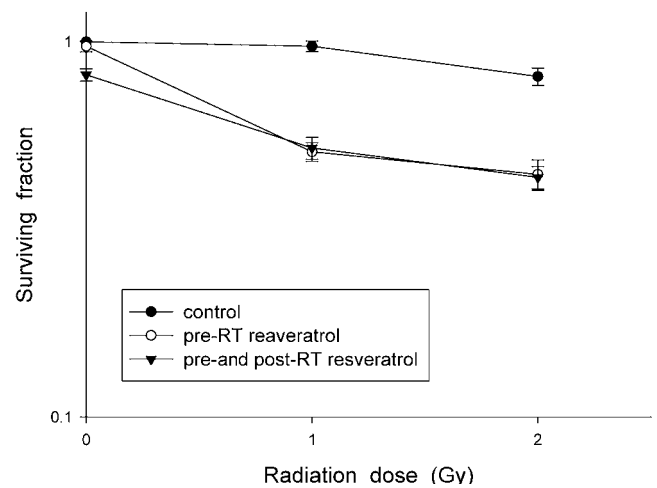


Fig. 4. Surviving fractions of NCI-H838 cells. Cells were treated with vehicle (●), resveratrol 25 μ M for 24 h with (▼) or without (○) subsequent removal of drug before radiation. Data from three separate experiments are presented as mean \pm SEM.

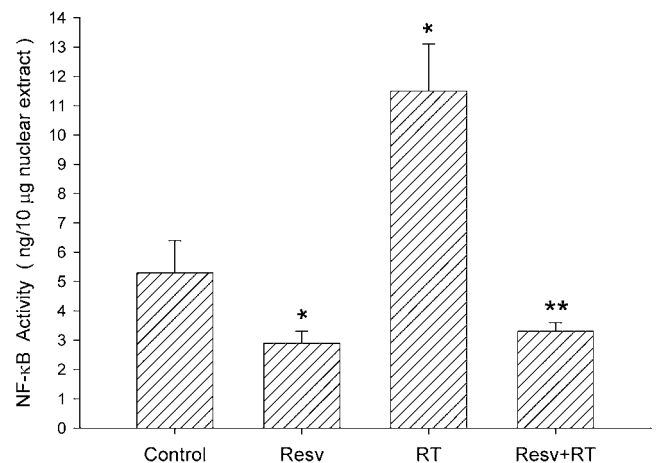


Fig. 5. The effects of radiation and resveratrol (Resv) on NF- κ B activity in NCI-H838 cells. Data from 3 separate experiments are expressed as the mean \pm SEM. * $p < 0.05$, compared with control group. ** $p < 0.05$ compared with RT alone.

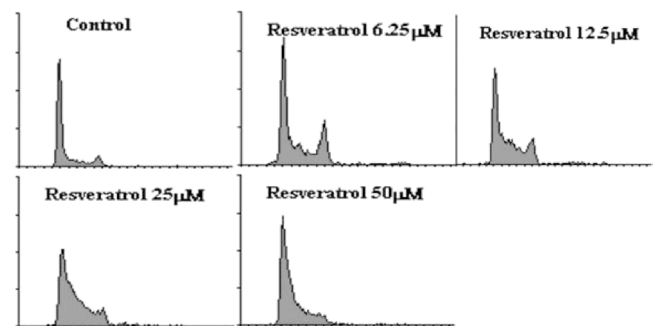


Fig. 6. Cell cycle distribution of NCI-H838 cells. After 24 hours' treatment with resveratrol (0 to 50 μ M), cells were harvested and analyzed for cell cycle distribution by using flow cytometry.

atrol 25 μM for 24 h followed by washing out and irradiation 2 Gy, or resveratrol 25 μM for 24 h followed by irradiation 2 Gy and washing out 24 h later. For nuclear staining, cells were washed with phosphate buffered saline, fixed with 75% alcohol, incubated with RNase (0.1 mg/mL) at 37°C for 30min, and stained with propidium iodide 0.5 mg/mL for 5min. The changes in nuclear morphology were observed under a fluorescent microscopy.

Statistics

Data were expressed as mean \pm standard error of the mean

Table 1. Cell cycle distribution of NCI-H838 cells after 24 hours' treatment with resveratrol.

	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
Control	54.9 \pm 1.6	26.4 \pm 4.3	18.7 \pm 2.5
Resveratrol 6.25 μM	47.2 \pm 6.4	32.5 \pm 4.5	20.3 \pm 3.9
Resveratrol 12.5 μM	43.4 \pm 6.2*	38.4 \pm 7.9	18.1 \pm 1.9
Resveratrol 25 μM	32.2 \pm 3.2*	54.4 \pm 6.8*	13.4 \pm 1.0*
Resveratrol 50 μM	31.5 \pm 2.0*	58.4 \pm 6.0*	10.1 \pm 2.4*

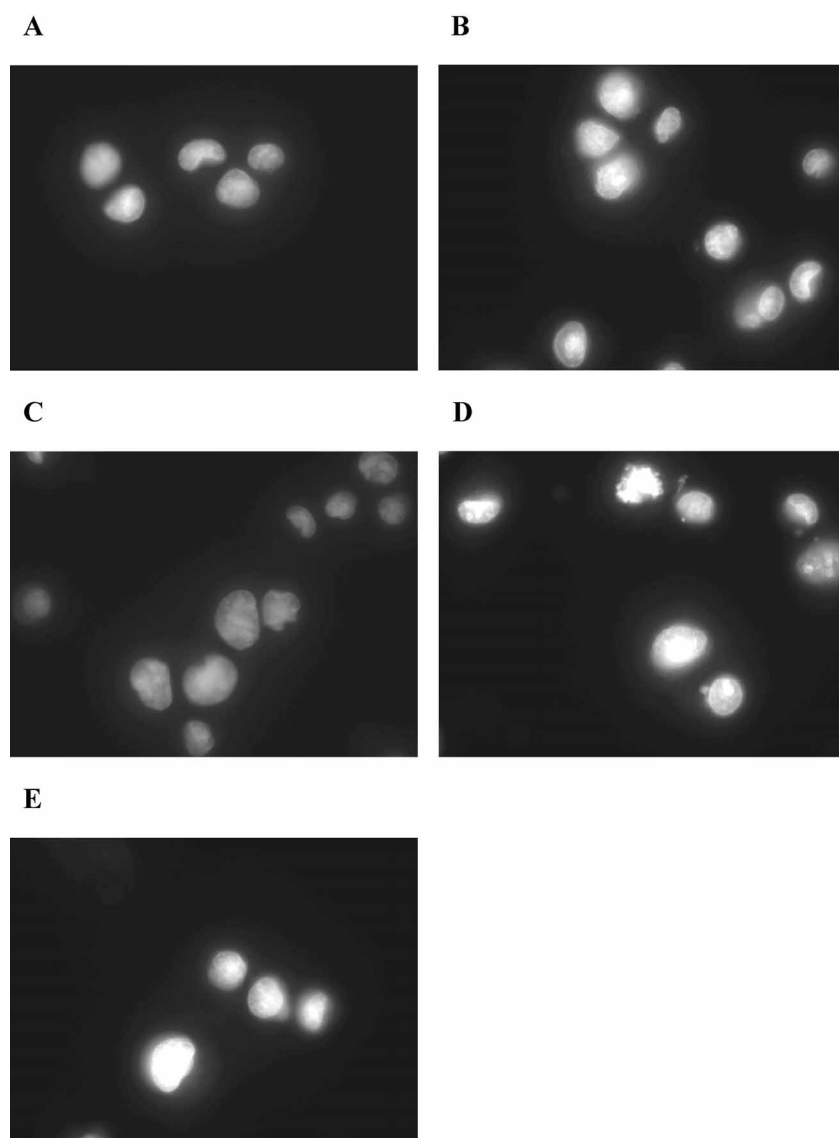


Fig. 7. The nuclear morphology of NCI-H838 cells. NCI-H838 cells were treated with vehicle (A), resveratrol 25 μM for 24 h (B), irradiation 2 Gy (C), resveratrol 25 μM for 24 h followed by washing out and irradiation 2 Gy (D), or resveratrol 25 μM for 24 h followed by irradiation 2 Gy and washing out 24 h later (E). Cells were fixed with 75% alcohol, incubated with RNase (0.1 mg/mL) at 37°C for 30min, and stained with propidium iodide 0.5 mg/mL for 5min. The nuclear morphology was observed and photographed under a fluorescent microscopy. Magnification 1000X.

(SEM). Analysis of variance was used to analyze the differences in NF- κ B activity and cell cycle distribution between various treated groups and controls. SPSS 10.0 software (SPSS Inc, Chicago) was used for analysis of all the data. Statistical significance was assumed where the p value was less than 0.05.

RESULTS

Effect of resveratrol treatment on viability of NCI-H838 cells

To evaluate the ability of resveratrol to sensitize tumor cells to radiation, non-toxic (cell viability >90%) doses of resveratrol were estimated before radiosensitization experiments in vitro. After 24 hours' exposure, the highest non-toxic dose of resveratrol for NCI-H838 cells was 25 μ M (Fig. 2).

Effect of resveratrol treatment on radiosensitivity of NCI-H838 cells

Radiation of untreated NCI-H838 cells at a dose of 2 Gy reduced the surviving fraction to 79.0%. Pretreatment with resveratrol at 12.5, 25, and 50 μ M markedly decreased the survival of irradiated tumor cells (Fig. 3). The SERs were 1.5, 2.2, and 2.5 for doses of 12.5, 25, and 50 μ M, respectively. The radiosensitizing effect of resveratrol (25 μ M) pretreatment only was similar to that of combination of pre-radiation (2 Gy) and post-radiation treatment (Fig. 4). However, the longer exposure of resveratrol (25 μ M) alone to NCI-H838 cells reduced their clonogenicity to 81.7% in comparison to untreated control.

Effect of resveratrol on DNA binding of NF- κ B in NCI-H838 cells

The NF- κ B activity of control H838 cells was 5.3 ± 1.1 ng/10 μ g nuclear extract. In comparison to control cells, radiation (2Gy) increased and resveratrol (25 μ M for 24 h) decreased NF- κ B activity to 11.5 ± 1.6 ($p < 0.05$) and 2.9 ± 0.4 ($p < 0.05$) ng/10 μ g nuclear extract, respectively. Pretreatment with resveratrol (25 μ M) 24-h before radiation reduced radiation-induced NF- κ B activation to a level of 3.3 ± 0.3 ng/10 μ g nuclear extract ($p < 0.05$) (Fig. 5).

Effect of resveratrol on cell cycle distribution in NCI-H838 cells

The effect of resveratrol on cell cycle distribution was evaluated under the same conditions affecting radiosensitization by a DNA histogram. The percentages of untreated exponentially growing NCI-H838 cells in G₀/G₁, S and G₂/M phases were $54.9\% \pm 1.6\%$, $26.5\% \pm 4.3\%$, and $18.7 \pm 2.5\%$, respectively. After 24 hours' treatment with resveratrol (25 to 50 μ M), the proportion in the G₀/G₁ phase was significantly decreased down to $31.5\% \pm 1.9\%$, whereas the percentage in the S phase was increased up to $58.4 \pm 6.0\%$ in a dose-dependent manner (Fig. 6 and Table 1). The

proportion of cells in the G₂/M phase remained unchanged by treated of 6.25–12.5 μ M resveratrol and significantly decreased when the concentration of resveratrol increased to 25–50 μ M. There was no evident sub-G₁ population of cells noted.

It shows that the effect of resveratrol 25 μ M on cell cycle distribution is reversible. The cell cycle distribution returned to untreated pattern completely at 24 h, but not 4 and 8 h, after removal of drug.

The effect of resveratrol and radiation on nuclei fragmentation

In NCI-H838 cells, there was no evident sub-G₁ population noted after 24-h treatment of resveratrol (0–50 μ M). To confirm that resveratrol enhanced radiosensitivity of NCI-H838 cells via non-apoptotic effect, we stained nuclei with propidium iodide and observed the changes under a fluorescent microscope. It shows no evident nuclear fragmentation after treatment of resveratrol (25 μ M) or radiation (2 Gy). Moreover, the resveratrol, neither pre-radiation nor pre-radiation and post-radiation treatment, induced the nuclear fragmentation at the condition having radiosensitizing effect (Fig. 7).

DISCUSSION

The present study was designed to evaluate the effects of resveratrol on the radiosensitivity of human non-small cell lung cancer cells. Our results demonstrate that resveratrol enhanced the radiosensitivity of NCI-H838 cells, accompanied by inhibition of NF- κ B and S-phase arrest.

NF- κ B is a transcription factor which targets genes involved in a diverse array of cellular functions, including apoptosis, proliferation, and inflammatory response.²³ Radiation activates NF- κ B in a variety of cancer cells, although the effect of this radiosensitization is dependent on the cancer cell lines used.²⁴ In our investigation, resveratrol decreased radiation-induced NF- κ B activity to a level lower than that in untreated controls, implying involvement of NF- κ B in the radiosensitizing effect of resveratrol in NCI-H838 cells.

Initial studies in synchronized Chinese hamster cells showed a differential response of the cells to radiation depending on which phase of the cell cycle they were in during irradiation. Generally, cells in the mitotic phase (M) are most sensitive, followed by G₂ cells, and then early G₁ cells. Resistance gradually increases as cells proceed through the late G₁ and S phases, reaching a maximum in late S phase.

In this study, resveratrol treatment resulted in decrease in cells in the G₀/G₁ phase and increased the proportion in the S phase without affecting G₂/M distribution. This implies that resveratrol is a potent radiosensitizer in tumor cells, albeit by a mechanism not involving G₂/M arrest. Hsieh et al. reported that in the presence of resveratrol, bovine pul-

monary artery endothelial cells accumulated in the S and G₂/M phases, an effect that involved up-regulation of p53 and p21^{WAF1/CIP1} expression.²⁵⁾ We noted no changes in G₁-S transition after resveratrol treatment, suggesting that p53 and p21^{WAF1/CIP1} have a limited role in the NCI-H838 cells used in our study. It is possible instead that up-regulation of cyclin E/cdk2 and/or down-regulation of cyclin A/cdk2 is involved in resveratrol-induced cell cycle perturbations.

NCI-H838 cell line was derived from metastatic lymph node of a male Caucasian patient with stage 3B lung adenocarcinoma. In NCI-H838 cells, one *PPP2R1B* allele has been shown altered in sequence at G₅₁ and the other allele has a possible deletion through loss of heterozygosity.²⁶⁾ The *PPP2R1B* gene is a putative tumor suppression gene encoding the β isoform of the A subunit of the serine/threonine phosphatase 2A. Whether this genetic alteration correlates to the radiosensitivity in NCI-H838 cells remains undetermined.

In the present study, we demonstrated that resveratrol can sensitize NCI-H838 cells to radiation. However, the effects of resveratrol on increasing radiosensitivity under hypoxic conditions and on the Ras radiation resistance pathway²⁷⁾ are not clear and remain to be determined.

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