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Resveratrol-Induced Cell Inhibition of Growth and Apoptosis in MCF7 Human Breast Cancer Cells Are Associated With Modulation of Phosphorylated Akt and Caspase-9

Yan Li,[1] Junye Liu,[1] Xinping Liu,[1] Kefei Xing,[1] Yun Wang,[2] Fuyang Li,[1] and Libo Yao*,[1]

[1]The Department of Biochemistry and Molecular Biology and The State Key Laboratory of Cancer Biology, The Fourth Military Medical University, Xi'an 710032, China, E-mail: bioyao@fmmu.edu.cn; and [2]Xi'an Greenpharma Natural Medicine Lab, Xi'an 710075, China

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Abstract

Resveratrol (*trans*-3,4N,-5-trihydroxystilbene), a phytoalexin present in grapes and red wine, is emerging as a natural compound with potential anticancer properties. Here we show that resveratrol affects the growth of human breast cancer cell lines MCF7, MDA-MB-231, SK-BR-3, and Bcap-37 in a dose-dependent manner and that MCF7 is the most sensitive among the four cell lines. MCF7 cells treated with resveratrol showed typical characteristics of apoptosis including the poly (ADP-ribose) polymerase cleavage, TdT-mediated dUTP nick end labeling–positive staining, and morphologic changes. Phosphorylation of the oncogene product Akt was significantly reduced followed by decreased phosphorylation and increased processing of pro-caspase-9 on resveratrol treatment. These results indicate that resveratrol seems to exert its growth-inhibitory/apoptotic effect on the breast cancer cell line MCF7 via the Akt-caspase-9 pathway.

Index Entries: Apoptosis; resveratrol; MCF7; Akt; caspase-9.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Breast cancer is rarely diagnosed in women younger than 25 years. Beyond that age, the incidence rises steadily, reaching a peak at about the age of menopause. About 1 in 10 women in the United States and Canada will develop breast cancer. This incidence is similar to that of many European countries. The treatment options of breast cancer include mastectomy or breast conservation therapy (chemotherapy, hormonal therapy, or radiation therapy). However, the pain of surgery and the side effects of conservation therapy are inevitable. New strategies to combat breast cancer, such as the use of chemopreventive agents, are being developed.

Resveratrol is a polyphenolic compound found in at least 72 plant species, especially in grape skin, and seems to have a wide spectrum of biologic activity *(1–3)*. Figure 1 shows the chemical structures of *trans*-resveratrol and *cis*-resveratrol. Previous studies have demonstrated the apoptotic effects of resveratrol in some tumor cell lines in vitro *(4,5)*, and it was shown to have an anticarcinogenic effect on a two-stage mouse skin cancer model *(6)*. In HL-60 leukemia cells, treatment with resveratrol led to growth inhibition, induction of apoptosis, S-G2-phase cell-cycle arrest, and myelomonocytic differentiation *(7,8)*. Resveratrol also inhibited proliferation in JB6 mouse epidermal, CaCo-2 colorectal, and A431 epidermoid carcinoma cell lines *(9–11)*. Resveratrol inhibits tumor formation in several animal models of carcinogenesis, including mouse 7,12-dimethylbenz(*a*)-anthracene/12-*O*-tetradecanoylphorbol-13-acetate-induced skin cancers *(6)*; azoxymethane-induced colon cancers *(12)*; and transplanted Yoshida rat ascites hepatomas *(13)*. The effects of resveratrol on breast cancer cell lines are controversial. Some investigators demonstrated an antiproliferative effect in the MCF7, MDA-MB-231, KPL-1, MKL-F, and T47D cell lines *(7,14,15)*, whereas others showed growth enhancement in T47D and MCF7 cells *(16,17)*. The latter effect appears to be owing to the potential estrogenic effect of resveratrol *(16–18)*. However, the precise mechanism of the antitumor effects of resveratrol and whether resveratrol can inhibit the growth of all human breast cancer cells are not well understood. More studies using both cellular and animal models are needed before such data would be applicable to human use.

The available evidence indicates that several apoptotic pathways can be triggered by resveratrol, and some survival pathways can be inhibited by it *(19)*. The complex sequence of events leading to apoptotic cell death is governed by an elaborate regulatory scheme involving the actions of both initiator and executioner proteases. The most intensively studied initiator caspase is caspase-9, an essential throughput element in the so-called intrinsic or mitochondrially gated pathway of apoptosis *(20)*. Caspase-9 is an upstream signaling molecule of caspase-3, and its activation is stimulated by Apaf-1/cytochrome-*c* and inhibited by activity of the oncogene product Akt. The phosphorylation of Akt is an essential step for growth factor–mediated inhibition of caspase activation. It was shown that Akt

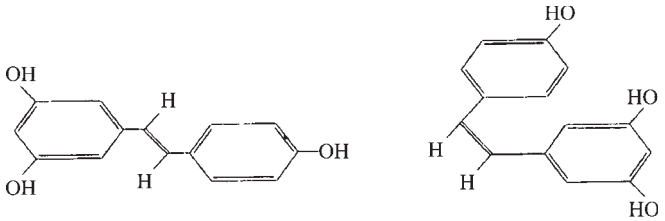

Fig. 1. Structure of *trans*-resveratrol and *cis*-resveratrol.

phosphorylated pro-caspase9 on serine-196 and resulted in the subsequent suppression of cytochrome-*c*-induced processing of pro-caspase9 *(21)*. However, a direct relationship between Akt/caspase-9 phosphorylation and the anticarcinogenic effect of resveratrol has not been observed so far.

The purposes of the present study were to determine whether resveratrol affects the growth of breast cancer cell lines through apoptosis, and if so, to examine the possible mechanisms involved.

Materials and Methods

Reagents

Resveratrol (trans-3,4,-5-trihydroxystilbene) (>99% pure) dissolved in 50% propanediol was provided by Xi'an Tianxingjian Natural Bio-products (Xi'an, China) and adjusted to final concentrations using complete RPMI-1640 medium. Fetal bovine serum (FBS) was purchased from the Yuan Heng Sheng Ma biotech institution (Beijing, China). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies (Beverly, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Hoechst 33258; 4',6-diamidino-2-phenylindole (DAPI); and the phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). The antibody of poly (ADP-ribose) polymerase (PARP) was from BD Pharmingen (San Diego, CA). The *in situ* apoptosis detection kit and the protease inhibitor mixture were from Roche (Penzberg, Germany). The bicinchoninic acid protein assay kit was purchased from Bio-Rad (Hercules, CA), and the enhanced chemiluminescence (ECL) kit was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies directed against total and phosphorylated forms of Akt were from Cell Signaling Technology (Beverly, MA). Total and phosphorylated forms of caspase-9 primary antibodies were from ProSci (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Cell Lines and Cell Culture

Human breast cancer cell lines MCF7, MDA-MB-231, SK-BR-3, and Bcap-37 were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in 5% CO_2 in RPMI-1640 or DMEM

supplemented with 10% FBS and 100 ng/mL each of penicillin and streptomycin.

Cell Growth Assay

The effects of resveratrol on cell growth were determined by MTT assay. Cells were seeded at 8×10^3 cells/well in 200 µL of medium in 96-well plates. After overnight incubation at 37°C in a humidified incubator, the cells were treated with resveratrol, ranging from 2 to 400 µmol/L for 24 h, and then 20 µL of MTT was added to each well and the cells were incubated for 4 h. To end the reaction, the culture supernatant was discarded, and 150 µL of dimethylsulfoxide was added to each well to dissolve the formazan crystals completely. Absorbance was recorded on a microplate reader at a wavelength of 490 nm.

Apoptotic Assay

Cell apoptotic status was analyzed by detecting the fragment of PARP cleavage by Western blot (see next section), TdT-mediated dUTP nick end labeling (TUNEL) staining *(22)*, and Hoechst 33258 staining *(23)*.

TUNEL staining was performed using an *in situ* apoptosis detection kit according to the manufacturer's instructions. Sections were then counterstained using DAPI and analyzed using a BX-60 fluorescent microscope (Olympus, Tokyo, Japan) at a magnification of ×200. Openlab software (Improvision) was used for image acquisition. For each slide, 10 fields were randomly chosen, and using a defined rectangular field area (×20 objective), a total of 100 cells were counted per field. The apoptotic index (AI) was calculated as follows: AI = number of TUNEL-positive cells/total number (DAPI positively stained cells) of MCF7 cells ×100%. The assays were performed in a double-blind manner.

Hoechst 33258 staining was done on glass cover slips. Cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature and stained with Hoechst 33258 (5 µg/mL) for 30 min at 37°C in the dark and washed twice with phosphate-buffered saline. The slides were examined on a BX-60 fluorescent microscope (Olympus) utilizing a 350-nm excitation and a 460-nm emission fluorescent filter. Apoptotic cells were identified by brightly staining condensed chromatin.

Western Blot

MCF7 cells were lysed with lysis buffer (10 mmol/L of Tris-HCl, pH 7.5; 1% NP-40; 0.1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 150 mmol/L of NaCl; 1 mmol/L of EDTA) containing protease inhibitor and phosphatase inhibitor cocktails. After centrifugation (12,000*g* for 30 min), the proteins were separated by SDS-polyacrylamide gel electrophoresis on polyacrylamide gels. Proteins were transferred electrophoretically onto a polyvinyl difluoride-nitrocellulose membrane. Blocking was performed in Tris-buffered saline containing 5% skim milk and 0.05%

Tween-20. Membranes were then incubated overnight with antibodies directed against β-actin (1:250), Akt (1:500), pS473-Akt (1:500), caspase-9 (1:500), and pS196-caspase-9 (1:500), followed by incubation with the corresponding secondary antibody (1:3000) at room temperature for 1 h. The blots were visualized with ECL-plus reagents according to the manufacturer's instructions.

Statistical Analysis

All values are reported as mean ± SE. Where appropriate, differences were compared using Student's *t*-tests or analyses of variance. Probability values of <0.05 were considered statistically significant. All statistical tests were performed with GraphPad Prism software version 4.0 (GraphPad Software, San Diego, CA).

Results

Resveratrol Shows Different Effects on Cell Growth at Different Concentrations

Figure 2 shows the effects of resveratrol on the growth of MCF7, MDA-MB-231, SK-BR-3, and Bcap-37 cells. Resveratrol inhibited tumor cell growth dramatically at concentrations of greater than 40 μmol/L (MCF7, SK-BR-3, and MDA-MB-231) and 80 μmol/L (Bcap-37), whereas cell growth was increased slightly at concentrations of 2 μmol/L (MCF7 and SK-BR-3), 2–20 μmol/L (MDA-MB-231), and 2–40 μmol/L (Bcap-37). The concentrations of resveratrol needed to inhibit cell growth of different human breast cancer cells were quite different. The IC_{50} values of resveratrol in MCF7, SK-BR-3, MDA-MB-231, and Bcap-37 were 65, 139, 207, and 213 μmol/L, respectively. The cell morphology of MCF7 was obviously changed with increasing concentrations of resveratrol (*see* supplemental figure), with a trend similar to that seen in the MTT assay. Therefore, MCF7 is the most sensitive cell line among the four human breast cancer cell lines.

Resveratrol Induced Apoptosis of MCF7

Resveratrol-induced apoptosis was observed by detecting an 85-kDa cleavage fragment of PARP (Fig. 3), and the positive TUNEL assay (Fig. 4) in MCF7 cells. After treatment with 200 μmol/L of resveratrol from 0 to 24 h, apoptotic cells were obviously increased. Hoechst 33258 staining of MCF7 cells treated with resveratrol also showed the appearance of characteristic apoptotic changes such as the condensation of nuclear chromatin (Fig. 5).

Involvement of Akt and Caspase-9 in Resveratrol-Induced MCF7 Cell Apoptosis

After incubation with resveratrol, phosphorylation on serine 473 of Akt was apparently reduced in MCF7 cells at 3 and 6 h without a change in

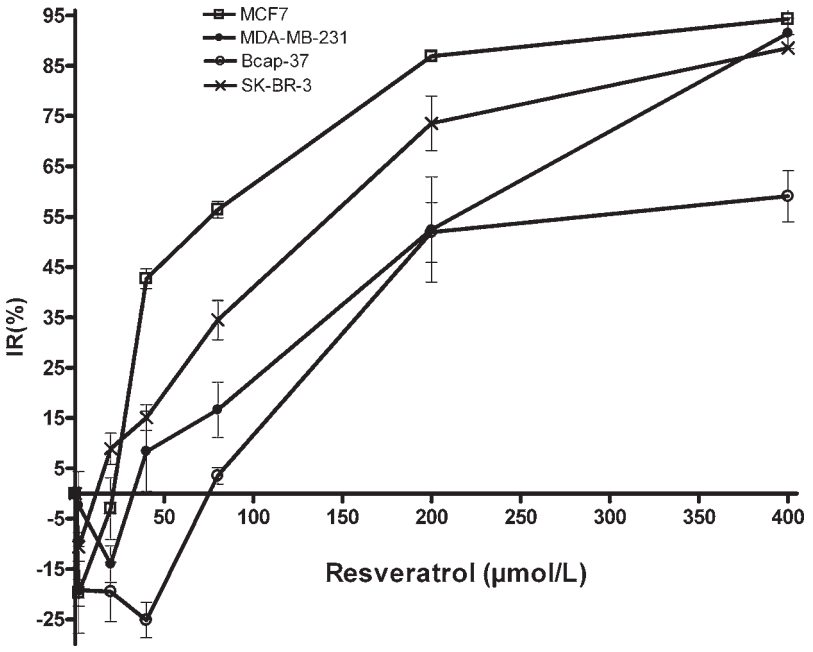

Fig. 2. Sensitivity of MCF7, MDA-MB-231, SK-BR-3, and Bcap-37 cells to resveratrol. Cells treated with increasing concentrations of resveratrol (2–400 μmol/L) for 24 h showed growth at lower concentrations and growth inhibition at higher concentrations. The inhibition rate (IR) was calculated by the following formula: IR = (1 – average OD_{490} of treated group / average OD_{490} of control) ×100%. The data shown are representative of at least three independent experiments.

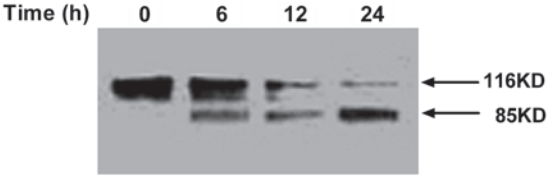

Fig. 3. Western blot analysis of PARP cleavage. MCF7 cells were treated with 200 μmol/L of resveratrol for 0, 6, 12, and 24 h. Immunoblotting was done with anti-PARP antibody.

protein levels, suggesting that resveratrol could inhibit the serine 473 phosphorylation of Akt (Fig. 6A). The level of Akt protein also started to decrease at 9 h. The effect of resveratrol treatment on the phosphorylation of caspase-9 on serine 196, which is known to be the site for Akt, was then assessed *(21)*. As expected, resveratrol treatment resulted in a decrease in phosphorylation of caspase-9 in MCF7 cells (Fig. 6A). Phosphorylation of Akt was inhibited by resveratrol as early as 3 h after resveratrol treatment, but a longer

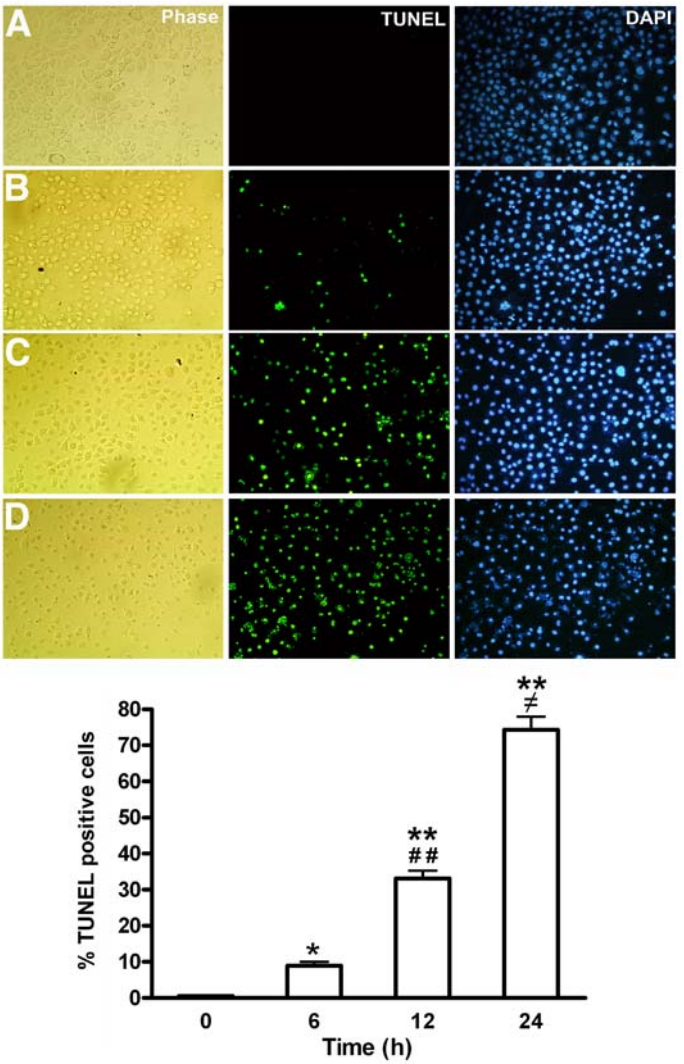

Fig. 4. Resveratrol caused an increase in TUNEL-positive cells. (*Top*) MCF7 cells were treated with 200 mmol/L of resveratrol. After 0 (row A), 6 (row B), 12 (row C), and 24 h (row D) of treatment, cells were stained using TUNEL or DAPI. Left-hand panels show phase images, middle panels show TUNEL fluorescence, and right-hand panels show the corresponding fields of DAPI fluorescence. (*Bottom*) The percentage of nuclear staining–positive cells for TUNEL in cell sections is shown. $^{*}p < 0.05$; $^{**}p < 0.01$ vs 0 h ; $^{\#\#}p < 0.01$ vs 6 h ; $p < 0.01$ vs 12 h. Original magnification: ×200.

period of time was needed to inhibit the phosphorylation of caspase-9 (9 h). The processing of caspase-9 was evaluated by detecting its 35-kDa cleavage fragment. It was found that the active form of caspase-9 increased as its phosphorylation decreased (Fig. 6B).

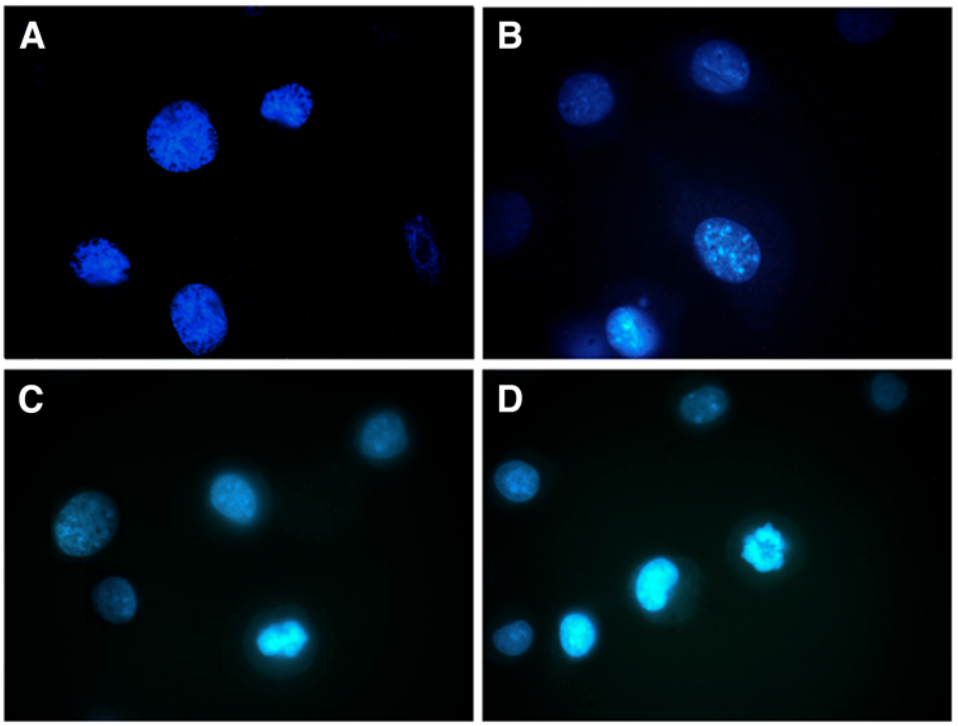

Fig. 5. Morphology of apoptotic cells visualized by Hoechst 33258 staining. MCF7 cells were treated with 200 μmol/L of resveratrol for **(A)** 0 h, **(B)** 6 h, **(C)** 12 h, and **(D)** 24 h. Original magnification: ×1000.

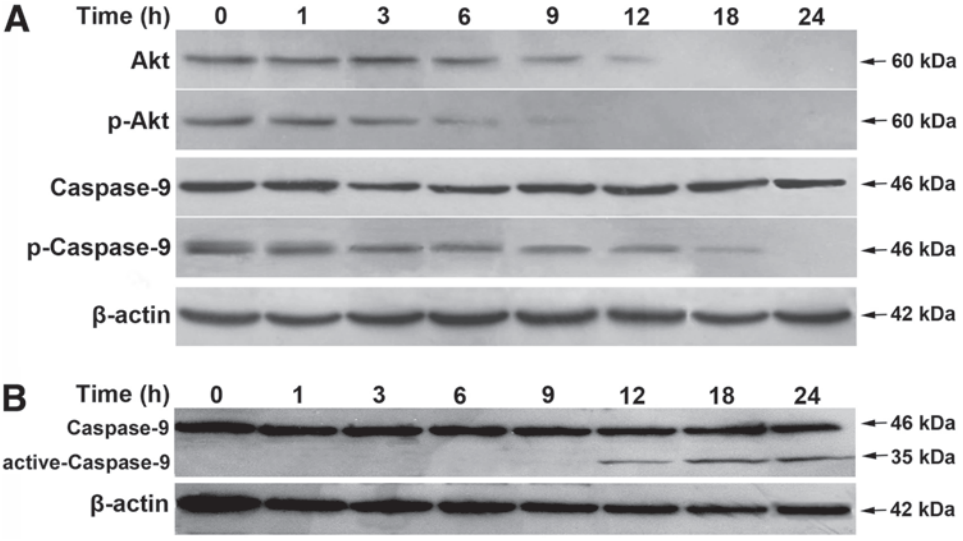

Fig. 6. Time-dependent effect of resveratrol on phosphorylation of Akt and caspase-9 **(A)** as well as active form of caspase-9 **(B)**. The data shown are from a representative experiment repeated three times with similar results.

Discussion

The inhibition of tumor formation by resveratrol is thought to occur at the stages of tumor initiation, promotion, and progression. In some tumor cell models, this tumor inhibition may involve induction of apoptosis *(7,9,24)*. We observed that resveratrol inhibited the growth of MCF7, MDA-MB-231, SK-BR-3, and Bcap-37 cells in a dose-dependent manner. The cytotoxic activities of resveratrol are variable in different types of human breast cancer cells. Among four human breast cancer cell lines examined, the results of the MTT assay showed that MCF7 exhibited the highest sensitivity, with an IC_{50} of 65 μmol/L.

In the present study, MCF7 cell death induced by resveratrol was attributed to apoptosis. After treatment with resveratrol, we observed the typical morphological characteristics of apoptosis, such as chromatin condensation and fragmentation. In addition, the 85-kDa cleavage fragment determined by the PARP cleavage assay and apoptosis-positive cells detected by the TUNEL assay clearly revealed that resveratrol rapidly induced apoptotic cell death in a time-dependent manner.

Various stimuli of apoptosis lead to the activation of a family of cysteine proteases with specificity for aspartic acid residues, referred to as caspases *(25)*. The activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and breaking down structural components of the cell *(26)*. The targets of such cleavage events include PARP and other caspases *(27,28)*. Caspase-9 binds to Apaf-1 and becomes activated under such condition. Activated caspase-9 in turn cleaves and activates caspase-3. An active-site mutant of caspase-9 blocks caspase-3 activation and cellular apoptosis in vivo *(29)*.

Previous studies have documented that resveratrol-induced apoptosis involved caspase activation in vitro *(30,31)*, which was further confirmed in the present examination of the effects of resveratrol on MCF7 cells. The caspases involved in cell death and the pathways leading to their activation could differ from one cell type to another. As the upstream molecule of caspase-3 and PARP, caspase-9 may play an important regulatory role in resveratrol-induced cell apoptosis. Our results showed that resveratrol treatment resulted in a time-dependent decrease in the phosphorylation of caspase-9. The reduction in caspase-9 phosphorylation should enhance its processing and initiate apoptosis. This is further confirmed here by the detection of a 35-kDa degradation product of caspase-9, which provided a new explanation for the role of resveratrol.

As the upstream kinase of caspase-9 as well as other important cell apoptosis regulatory protein, Akt is considered as a crucial molecule for cell survival *(32)*. Here we provided the first evidence that Akt may serve as the potential target of resveratrol. However, the data cannot determine whether resveratrol acts on Akt directly or through its upstream molecules, such as phosphoinositide 3-kinase or phosphoinositide-dependent kinase-1; therefore, further investigations are necessary. The level of Akt protein was

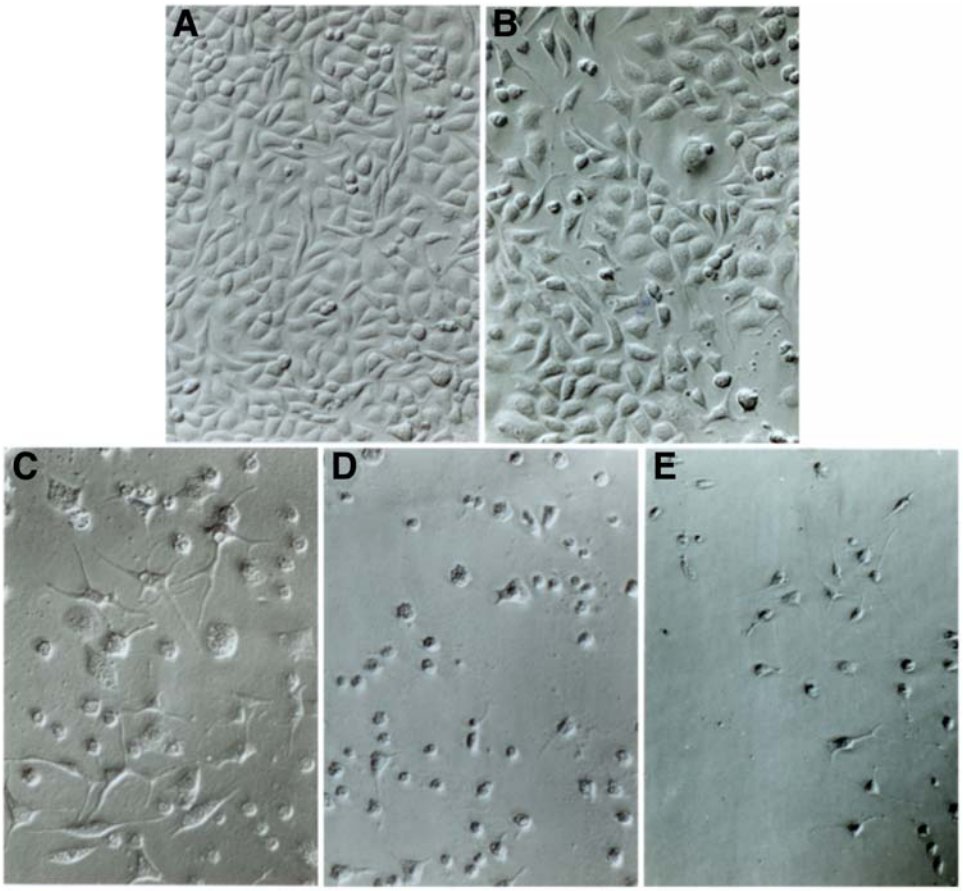

Figure Supplement. Change in cell morphology of MCF7 cells on resveratrol treatment. The cells were incubated with **(A)** 1% propanediol, the solvent control; or **(B)** 20, **(C)** 40, **(D)** 80, and **(E)** 200 µmol/L of resveratrol for 24 h. The photos were taken with an Olympus phase contrast microscope. Original magnification: ×200.

not changed in MCF7 cells at 3 or 6 h, when a significant decrease in phosphorylation of Akt was detected; however, Akt protein started to decrease at 9 h. The reduction in the amount of Akt protein beginning at 9 h could be owing to the change in Akt protein or mRNA stability or *AKT* gene expression during treatment of the cells with resveratrol. It will be interesting to explore further the features and mechanism of such a regulatory role of resveratrol.

We found that resveratrol has different effects on cell growth at different concentrations. Resveratrol inhibited tumor cell growth dramatically at concentrations of greater than 40 µmol/L (MCF7, SK-BR-3, and MDA-MB-231) and 80 µmol/L (Bcap-37), whereas cell growth increased slightly at less than 2 µmol/L (MCF7 and SK-BR-3), 20 µmol/L (MDA-MB-231), and 40 µmol/L (Bcap-37). These findings are consistent with those reported by Gehm et al. *(16)* in 1997. This phenomenon has been referred to as

"hormesis," a term describing the stimulus actions of a substance at low concentrations and inhibitory actions at higher concentrations *(33)*. More studies of this phenomenon are required before considering resveratrol as a chemotherapeutic drug for breast cancer.

Conclusion

Resveratrol exerts potent cytotoxic activity and induces apoptosis in MCF7 cells. Phosphorylation of Akt on serine 473 decreases on resveratrol treatment, which may be responsible for the decreased phosphorylation and enhanced processing of caspase-9. Here, at least in part, we have provided a new explanation for apoptosis induced by resveratrol.

Acknowledgments

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