Resveratrol Causes WAF-1/p21-mediated G_1-phase Arrest of Cell Cycle and Induction of Apoptosis in Human Epidermoid Carcinoma A431 Cells[1]

Nihal Ahmad,[2] Vaqar M. Adhami, Farrukh Afaq, Denise K. Feyes, and Hasan Mukhtar

Department of Dermatology, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio 44106

ABSTRACT

Resveratrol (*trans*-3,4′,5,-trihydroxystilbene), a phytoalexin found in grapes, nuts, fruits, and red wine, is a potent antioxidant with cancer-preventive properties. The mechanism by which resveratrol imparts cancer chemopreventive effects is not clearly defined. Here, we demonstrate that resveratrol, via modulations in cyclin-dependent kinase (cdk) inhibitor-cyclin-cdk machinery, results in a G_1-phase arrest of the cell cycle followed by apoptosis of human epidermoid carcinoma (A431) cells. Resveratrol treatment (1–50 μM for 24 h) of A431 cells resulted in a dose-dependent (*a*) inhibition of cell growth as shown by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, (*b*) G_1-phase arrest of the cell cycle as shown by DNA cell cycle analysis, and (*c*) induction of apoptosis as assessed by ELISA. The immunoblot analysis revealed that resveratrol treatment causes a dose- and time-dependent (*a*) induction of WAF1/p21; (*b*) decrease in the protein expressions of cyclin D1, cyclin D2, and cyclin E; and (*c*) decrease in the protein expressions of cdk2, cdk4, and cdk6. Resveratrol treatment was also found to result in a dose- and time-dependent decrease in kinase activities associated with all of the cdks examined. Taken together, our study suggests that resveratrol treatment of the cells causes an induction of WAF1/p21 that inhibits cyclin D1/D2-cdk6, cyclin D1/D2-cdk4, and cyclin E-cdk2 complexes, thereby imposing an artificial checkpoint at the $G_1 \rightarrow S$ transition of the cell cycle. This series of events results in a G_1-phase arrest of the cell cycle, which is an irreversible process that ultimately results in the apoptotic death of cancer cells. To our knowledge, this is the first systematic study showing the involvement of each component of cdk inhibitor-cyclin-cdk machinery during cell cycle arrest and apoptosis of cancer cells by resveratrol.

INTRODUCTION

Chemoprevention, which refers to the administration of synthetic or naturally occurring agents to prevent the initiation and/or promotional events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasia (1–5). In this regard, several naturally occurring antioxidants are showing promise (1–5). Resveratrol (*trans*-3,4′,5,-trihydroxystilbene; Fig. 1), a phytoalexin found in grapes, nuts, fruits, and red wine, is a potent antioxidant (6–9) with anti-inflammatory (10) and cancer-preventive (10–13) properties. Traditional Japanese and Chinese folk medicines have used a root extract of the weed *Polygonum cuspidatum*, which contains resveratrol, to fight liver, skin, and circulatory diseases (10). Epidemiological studies have indicated that certain populations, *e.g.,* the French and the Greek populations, have a lower risk of cardiovascular diseases despite consuming a diet relatively rich in fat (13). This lower risk of heart diseases was proposed to be associated with a regular but moderate consumption of red wine by these populations, and this phenomenon was dubbed the French paradox (13).

The anticancer properties of resveratrol were not appreciated until 1997, when this compound was shown to be a potent cancer chemopreventive agent in assays representing all of the three major stages of carcinogenesis (11). In this study, resveratrol was found to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and tumorigenesis in a two-stage mouse skin tumorigenesis model (11). Furthermore, it was also demonstrated that topical application of resveratrol resulted in a significant inhibition of 7,12-dimethylbenz(*a*)anthracene-initiated and 12-*O*-tetradeca-noylphorbol-13-acetate-promoted formation of skin tumors in CD-1 mice (11). This led to the initiation of investigations on the cancer chemopreventive effects of resveratrol. However, the mechanism(s) by which resveratrol imparts its cancer chemopreventive effects is not clearly defined.
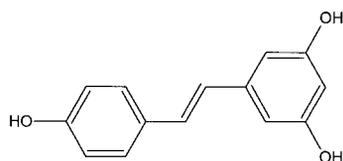
Many studies have shown an association between cell cycle regulation and cancer, and in recent years, inhibition of the cell cycle has come to be appreciated as a target for the management of cancer (14–19). Anticancer agents may alter regulation of the cell cycle machinery, resulting in an arrest of cells in different phases of the cell cycle and thereby reducing the growth and proliferation of cancerous cells. Furthermore, in recent years, programmed cell death, *i.e.*, apoptosis, which is a phenomenon associated with many physiological and pathological processes including cancer, has come to be appreciated as an ideal way to eliminate precancerous and/or cancer cells (Refs. 20–23 and the references therein). Thus, chemopreventive agents that can modulate apoptosis may be able to affect the steady-state cell population, which may be useful in the management and therapy of cancer. In recent years, many studies have shown an association of cell cycle regulation and apoptosis with cancer, inas-

Structure of *trans*-Resveratrol
(*trans*-3,4',5,-trihydroxystilbene)

Fig. 1 Structure of *trans*-resveratrol.

much as the cell cycle inhibitors and apoptosis-inducing agents are being appreciated as weapons for the management of cancer (14–24).

In eukaryotes, regulation of the cell cycle is controlled in part by a family of protein kinase complexes, and each complex is composed minimally of a catalytic subunit, the cdk,[3] and its essential activating partner, the cyclin (15, 19, 25–28). These complexes are activated at specific intervals during the cell cycle but can also be induced and regulated by exogenous factors (15, 19, 25–28). The cyclin-cdk complexes are subjected to inhibition via binding with a class of proteins known as ckis (15, 18, 19, 25–28). Recent studies have suggested that cell cycle arrest in malignant cells is often associated with apoptotic cell death (20, 21, 29–32).

In the present study, we demonstrate that resveratrol, via modulations in the cki-cyclin-cdk machinery, results in a G_1-phase arrest of the cell cycle followed by apoptosis of human epidermoid carcinoma A431 (a keratinocytic carcinoma cell line) cells. To our knowledge, this is the first systematic study showing the involvement of each component of the cki-cyclin-cdk machinery during cell cycle arrest and apoptosis of cancer cells by resveratrol.

MATERIALS AND METHODS

Reagents. Resveratrol (>99% pure) was purchased from Sigma Chemical Co. (St. Louis, MO). All of the antibodies were purchased from Lab Vision Corp. (Fremont, CA) unless stated otherwise. The DC Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Novex precast Tris-glycine gels and [γ-^{32}P]ATP (6000 Ci/mmol) were obtained from Amersham Life Sciences, Inc. (Arlington Heights, IL).

Cell Culture. Human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment.

Treatment of Cells. Resveratrol (dissolved in DMSO) was used for the treatment of cells. For dose-dependent studies, the cells (at 70–80% confluence) were treated with resveratrol

at 1, 5, 10, 25, and 50 μM (unless stated otherwise) for 24 h in complete cell medium, whereas for time-dependent studies, the cells (at 60–70% confluence) were treated with a 10 μM dose of resveratrol for 1, 3, 6, 12, 24, and 48 h. The control cells were incubated with the highest amount of DMSO used for 24 h for the dose-dependent study. For time-dependent study, in addition to the 0 h control, we also included a control treated with DMSO for 48 h because it was the longest time point postresveratrol administration in our experimental protocol.

Cell Growth/Cell Viability and DNA Cell Cycle Analysis. The effect of resveratrol on the viability of cells was determined by the MTT assay. The cells were plated at 2×10^5 cells/well in 200 μl of DMEM complete medium containing 1, 5, 10, 25, and 50 μM concentrations of resveratrol in 96-well microtiter plates. Each concentration of resveratrol was repeated in 10 wells. After incubation for 24 h at 37°C in a humidified incubator, cell viability was determined using the MTT assay. Briefly, 4 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for 2 h, and then the plate was centrifuged at 1800 rpm for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. Formazan crystals were dissolved in 150 μl of DMSO. The absorbance was recorded on a microplate reader at a wavelength of 540 nm. The effect of resveratrol on growth inhibition was assessed as the percentage of cell viability (vehicle-treated cells were taken as 100% viable).

For DNA cell cycle analysis, the cells were treated with vehicle alone (similar volumes of DMSO) or resveratrol (1, 5, 10, 25, and 50 μM) for 24 h. Untreated cells were also included in this experiment for comparison. After treatments, the cells were collected by trypsinization, washed with cold PBS [10 mM (pH 7.4)], and resuspended in 50 μl of cold PBS. The cells were fixed in 450 μl of ice-cold methanol by incubating them for 1 h at 4°C. The cells were then centrifuged at 1100 rpm for 5 min, and the pellet was washed twice with cold PBS, suspended in 500 μl of PBS, and incubated with RNase (20 μg/ml, final concentration) for 30 min at 37°C. The cells were then chilled over ice for 10 min, stained with propidium iodide (50 μg/ml, final concentration) for 1 h, and analyzed by flow cytometry.

Measurement of Apoptosis by ELISA. The induction of apoptosis by resveratrol was assessed using the Cell Death Detection ELISA^{PLUS} kit obtained from Roche Molecular Biochemicals USA (Indianapolis, IN). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after apoptotic cell death. For determination of apoptosis by ELISA, the A431 cells (1×10^4 cells/well) were treated with resveratrol at 1, 5, 10, 25, and 50 μM concentrations for 24 h in a 96-well plate. Each concentration of resveratrol was repeated in 10 wells. The induction of apoptosis was evaluated by assessing the enrichment of nucleosomes in the cytoplasm and determined exactly as described in the manufacturer's protocol.

Preparation of Cell Lysates and Immunoblot Analysis. For the immunoblot analysis in our dose-dependent study, we used 1, 5, 10, and 25 μM concentrations of resveratrol because we found that the highest concentration of resveratrol (50 μM) used in our study was associated with very low cell viability and massive apoptosis. However, for the time-dependent study, the similar protocol described earlier was followed. After the treat-

---

[3] The abbreviations used are: cdk, cyclin-dependent kinase; cki, cyclin-dependent kinase inhibitor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

ments, the medium was aspirated, and the cells were washed twice with cold PBS [10 mM (pH 7.4)]. The cells were incubated with ice-cold lysis buffer [50 mM Tris-HCl, 150 nm NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP40, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor mixture (Protease Inhibitor Cocktail Set III; Sigma Chemical Co.) for 30 min over ice. Then, the cells were scraped, and the lysate was collected in a microfuge tube and passed through a 21-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at $14,000 \times g$ for 15 min at 4°C, and the supernatant (total cell lysate) was either used immediately or stored at −70°C. The protein concentration was determined by using the DC Bio-Rad assay (Bio-Rad Laboratories) as per the manufacturer's protocol.

For immunoblot analysis, 25–50 μg of protein were resolved over 12% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked with blocking buffer [5% nonfat dry milk in 1% Tween 20 in 20 mM Tris-buffered saline (pH 7.5)] by incubating it for 1 h at room temperature followed by incubation with the appropriate primary antibody (at the dilutions recommended by the manufacturer) in blocking buffer overnight at 4°C. This was followed by incubation with the appropriate secondary antibody horseradish peroxidase conjugate (Amersham Life Sciences, Inc.), and the protein expression was detected by chemiluminescence using an enhanced chemiluminescence kit (Amersham Life Sciences, Inc.) and autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, NY). For every immunoblot, equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody.

Kinase Activity Assay. The cells were treated and lysed as described previously, except that lysis buffer was replaced with kinase lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM NaF, 1.0 mM EDTA, 1.0 mM EGTA, 1% Triton X-100, 0.5% NP40, and freshly added phenylmethylsulfonyl fluoride (0.01%) and protease inhibitor mixture. The cyclin-cdk complexes were immunoprecipitated as described above using the appropriate primary antibody raised to the COOH terminus of the protein. For immunoprecipitation, cell lysates containing 200 μg of protein were placed in 1.0 ml of kinase lysis buffer and precleared by incubation with normal IgG (1 μg) and protein A-Sepharose 4B fast flow (20 μl; Sigma Chemical Co.) for 1 h at 4°C. The supernatant was collected and incubated with 1.0 μg of the appropriate primary antibody and 20 μl of protein A-Sepharose overnight at 4°C. The immunocomplexes were washed three times with the kinase lysis buffer and once with kinase buffer containing 50 mM Tris (pH 7.4), $MgCl_2$ (10 mM), and DTT (1 mM). The beads were then incubated with 40 μl of "hot" kinase solution containing 5.0 μg of histone H1 (for cdk2-associated kinase activity) or pRb-GST (for cdk4- and cdk6-associated kinase activities), 80 μM ATP, and 5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Life Sciences, Inc.) in kinase buffer for 30 min at 37°C. The sample buffer (5×, 8 μl) was added to the tubes, which were then heated at 100°C for 5 min over boiling water. The samples were resolved over a 12% polyacrylamide gel. The gels were dried using a Bio-Rad gel drier (Model 583; Bio-Rad Laboratories)

followed by detection by autoradiography with XAR-5 film (Eastman Kodak Co.).

RESULTS

Resveratrol Causes Cell Growth Inhibition, G_1-phase Arrest, and Apoptosis in A431 Cells. Our aim was to investigate whether resveratrol treatment imparts antiproliferative effects against skin cancer cells. Therefore, using human epidermoid carcinoma A431 cells, we first evaluated the effect of resveratrol on the growth of these cells by MTT assay. As shown in Fig. 2A, resveratrol treatment (1–50 μM for 24 h) of A431 cells resulted in a dose-dependent inhibition of cell growth.

To assess whether resveratrol-induced growth inhibition of the cells is mediated via alterations in cell cycle regulation and apoptosis, we evaluated the effect of resveratrol on cell cycle distribution and fragmentation of DNA. First, we performed DNA cell cycle analysis using growing A431 cells, and resveratrol treatment was found to result in a significant dose-dependent increase of cell population in the G_1 phase of the cell cycle in A431 cells (49%, 55%, 63%, 75%, and 78% cells at 1, 5, 10, 25, and 50 μM concentrations of resveratrol, respectively; Fig. 2B). In this experiment, the population of vehicle (similar volumes of DMSO)-treated cells was not found to be affected as compared with the untreated cells. The G_1-phase population of vehicle-treated cells was found to range between 41% and 47% (data not shown), probably because we used growing (unsynchronized) cells in these experiments. The increase in cell population in the G_1 phase was found to be associated with a concomitant decrease in cell population in the G_2 phase, whereas the population of cells in M phase did not change significantly as compared with the corresponding controls.

Next, using ELISA, we characterized the enrichment of nucleosomes in the cytoplasm of the cells, which is indicative of induction of apoptosis. As shown by the data in Fig. 2C, compared with vehicle-treated control, resveratrol treatment of A431 cells resulted in significant dose-dependent apoptosis, reaching a maximum at a 25 μM concentration of resveratrol. However, at the higher concentration of 50 μM, a slight decrease in apoptosis was observed as compared with the 25 μM dose of resveratrol. This decrease is probably a result of massive loss of the cells, which could not be evaluated for nucleosome enrichment. For this reason, in our additional experiments, we did not use this concentration of resveratrol. Furthermore, induction of apoptosis was also evident from the immunoblot analysis of PARP. As shown in Fig. 2D, resveratrol treatment of A431 cells resulted in a dose-dependent cleavage of PARP, which is indicative of induction of apoptosis.

Resveratrol-induced Cell Cycle Arrest and Apoptosis Is Mediated via an Induction in WAF1/p21 and Consequent Inhibition in Cyclins D1, D2, and E and cdk2, cdk4, and cdk6. Because our studies demonstrated that resveratrol treatment of A431 cells results in a G_1-phase cell cycle arrest and apoptosis of A431 cells, we examined the effect of resveratrol on cell cycle-regulatory molecules operative in the G_1 phase of the cell cycle. We assessed the effect of resveratrol on the induction of WAF1/p21, which is known to regulate the entry of cells at the G_1-S-phase transition checkpoint and induce apo-
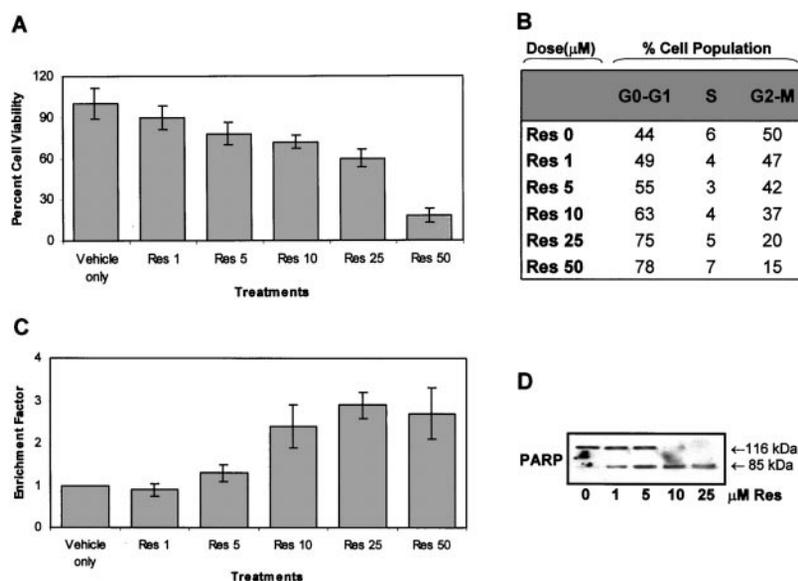
Fig. 2 *A,* effect of resveratrol on cell growth. As detailed in "Materials and Methods," the cells were treated with resveratrol, and the viability of cells was determined by MTT assay. The data are expressed as the percentage of cell viability and represent the means ± SE of three experiments in which each treatment was performed in multiple wells. *B,* effect of resveratrol on cell cycle distribution. The cells were treated with vehicle or resveratrol as detailed in "Materials and Methods" and analyzed by flow cytometry. The percentage of cells in G_0-G_1, S, and G_2-M phase were calculated using Cellfit computer software and are represented within the histograms. The data shown here are from a representative experiment repeated three times with similar results. *C,* effect of resveratrol on the enrichment of nucleosomes in the cytoplasm. As detailed in "Materials and Methods," the A431 cells (1×10^4 cells/well) were treated with specified concentrations of resveratrol in a 96-well plate, and the enrichment of nucleosomes in the cytoplasm was determined using the Cell Death Detection ELISA[PLUS] kit exactly as described in the manufacturer's protocol. The data presented are the means ± SE of three experiments in which each treatment was performed in multiple wells. *D,* effect of resveratrol on cleavage of PARP. The cells were treated with vehicle only or specified concentrations of resveratrol for 24 h and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by Western blot analysis using anti-PARP antibody obtained from Upstate Biotechnology, Inc. (Waltham, MA) and chemiluminescence detection. The details are described in "Materials and Methods." The data shown here are from a representative experiment repeated three times with similar results.
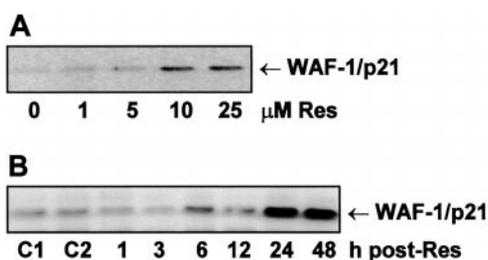
Fig. 3 Effect of resveratrol on protein expression of WAF1/p21. *A,* dose-dependent effect; *B,* time-dependent effect. As detailed in "Materials and Methods," the cells were treated with vehicle only or specified concentrations of resveratrol and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. The data shown here are from a representative experiment repeated three times with similar results.

ptosis. Immunoblot analysis revealed that resveratrol treatment of the cells resulted in a significant dose- and time- dependent induction of WAF1/p21 compared with the basal levels (Fig. 3).

Using immunoblot analysis, we also assessed the effect of resveratrol treatment on the protein expressions of the cyclins and cdks, which are known to be regulated by WAF1/p21. Resveratrol treatment of the cells resulted in a dose- and time-dependent decrease in protein expressions of cyclin D1, cyclin D2, and cyclin E (Fig. 4) as well as cdk2, cdk4, and cdk6 (Fig. 5). In the dose-dependent study, the decrease in cyclin D1 and cyclin D2 proteins was more pronounced than that of cyclin E. Similarly, in a dose-dependent study, the decrease in the protein expression of cdk4 and cdk6 was found to be more pronounced than that of cdk2 (Fig. 5). In the time-dependent study, however, the levels of cyclin D1 were found to be inhibited by resveratrol (10 μM) treatment as early as 6 h after treatment, but the inhibitory effect of the same treatment on cyclin D2 and cyclin E was evident only at 24 h after treatment (Fig. 4).

Resveratrol Treatment of A431 Cells Results in a Decrease in the Kinase Activities Associated with cdks. Kinase activities associated with the cdks are the driving force for progression of the cell cycle through the transition checkpoints because they activate the cyclins, the essential component of cyclin-cdk complexes; therefore, we assessed the effect of resveratrol treatment on the kinase activities associated with cdk2, cdk4, and cdk6. The radioactive kinase activity assay (Fig. 6) demonstrated that resveratrol treatment resulted in a dose- and time-dependent decrease in kinase activities associated with all of the cdks examined, although the extent of the effect differed. In the dose-dependent study, resveratrol treatment appears to affect the kinase activities of cdk2 and cdk4 in a much more pronounced fashion as compared with that of cdk6. Furthermore, the low dose of resveratrol (10 μM) also resulted in a significant inhibition of the kinase activities associated with
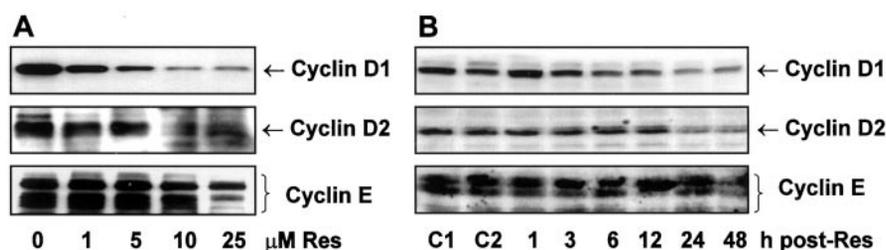
Fig. 4 Effect of resveratrol on protein expression of cyclin D1, cyclin D2, and cyclin E. *A,* dose-dependent effect; *B,* time-dependent effect. As detailed in "Materials and Methods," the cells were treated with vehicle only or specified concentrations of resveratrol and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. The data shown here are from a representative experiment repeated three times with similar results.

Fig. 5 Effect of resveratrol on protein expression of cdk2, cdk4, and cdk6. *A,* dose-dependent effect; *B,* time-dependent effect. As detailed in "Materials and Methods," the cells were treated with vehicle only or specified concentrations of resveratrol and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by Western blot analysis and chemiluminescence detection. The data shown here are from a representative experiment repeated three times with similar results.
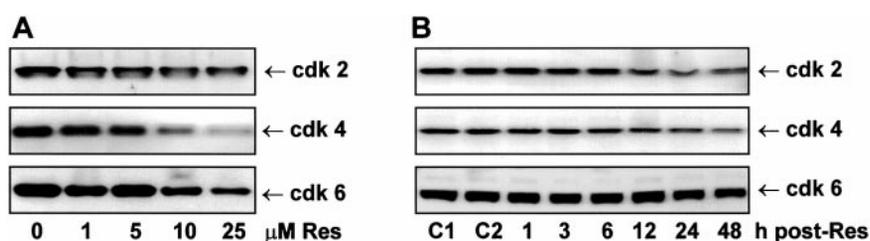
cdk2, cdk4, and cdk6 as early as 12, 24, and 6 h after treatment, respectively.

DISCUSSION

In recent years, naturally occurring antioxidant compounds present in the diet and beverages consumed by humans have gained considerable attention as cancer chemopreventive agents (1–5). Resveratrol, a polyphenolic compound present in grapes, nuts, fruits, and red wine, is a potent antioxidant with anti-inflammatory and cancer-preventive properties (7, 8, 10–13, 33–42). *In vitro* as well as *in vivo* studies have suggested that resveratrol may impart chemopreventive effects against certain cancers including skin cancer (10–13, 33–42). The molecular mechanism(s) by which resveratrol imparts cancer chemopreventive properties is not well defined. The present study was designed to define the mechanism(s) of the antiproliferative effects of resveratrol because it may result in the design of novel approaches for the management of cancer. In this study, we investigated the involvement and mechanism of cell cycle dysregulation and apoptosis during cell growth inhibition by resveratrol in skin cancer-derived cells, *i.e.,* human epidermoid carcinoma (A431) cells. The hypothesis that was tested in this study was whether resveratrol will impart an antiproliferative effect via a modulation of cki-cyclin-cdk machinery-mediated cell cycle dysregulation and apoptosis.

In our experiments, we used A431 cells, which are known to have nonfunctional (mutated) p53 carrying a G→A mutation at codon 273, resulting in an arginine to histidine substitution (43). We chose these cells because of the following facts. First, A431 cells are keratinocytic carcinoma cells, and our aim was to

investigate the mechanism of antiproliferative response of resveratrol against skin cancer because studies have shown that resveratrol is effective against skin carcinogenesis (11). Second, the tumor suppressor gene p53 is regarded as a key element in maintaining a balance between cell growth and cell death in the living system (29, 44, 45). p53, in response to DNA damage, triggers a variety of cell cycle-regulatory events to limit the proliferation of damaged cells (29, 44, 45). In a number of human tumors, including skin cancer, this "gatekeeper gene" is inactivated by mutation that results in unlimited cellular proliferation (29, 44, 45). Therefore, it is important to study the mechanism(s) by which resveratrol imparts antiproliferative effects in the absence of active (wild-type) p53.

Our data demonstrated that resveratrol treatment of the A431 cells resulted in significant (*a*) cell growth inhibition, (*b*) G_1-phase cell cycle arrest, and (*c*) apoptosis in a dose-dependent fashion. This is an important observation because the regulation of cell cycle and apoptotic machinery is important in the growth and development of neoplasms, and in recent years, cell cycle and apoptosis have become being increasingly appreciated as targets for intervention against cancer (4, 14–24). The observed induction of apoptosis by resveratrol is important because apoptosis is a physiological process that functions as an essential mechanism of tissue homeostasis and is regarded as the preferred way to eliminate unwanted cells (4). A wide variety of chemotherapeutic agents currently being considered for the management of cancer are known to kill the cells by mechanisms other than apoptosis, which is probably not a preferable way to manage cancer cell growth (4, 20–22).

Because many studies from this and other laboratories have
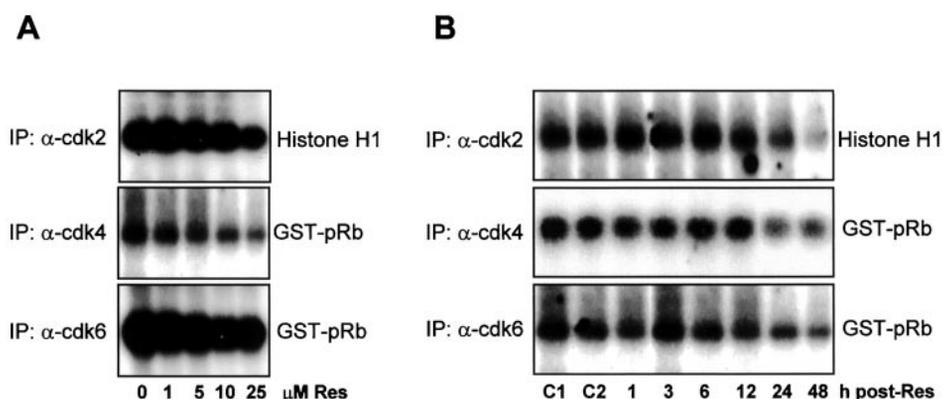
Fig. 6 Effect of resveratrol on kinase activities associated with cdk2, cdk4, and cdk6. *A,* dose-dependent effect; *B,* time-dependent effect. The cells were treated with vehicle only or resveratrol and harvested, and lysates were prepared using kinase lysis buffer. Protein (200 μg) was immunopre-cipitated in each case, and kinase activities were measured using the appropriate substrates and $[\gamma\text{-}^{32}P]ATP$ using gel electrophoresis as detailed in "Materials and Methods." The data shown here are from a representative experiment repeated twice with similar results.

Fig. 7 Proposed model for resveratrol-mediated cell cycle dysregulation and apoptosis.
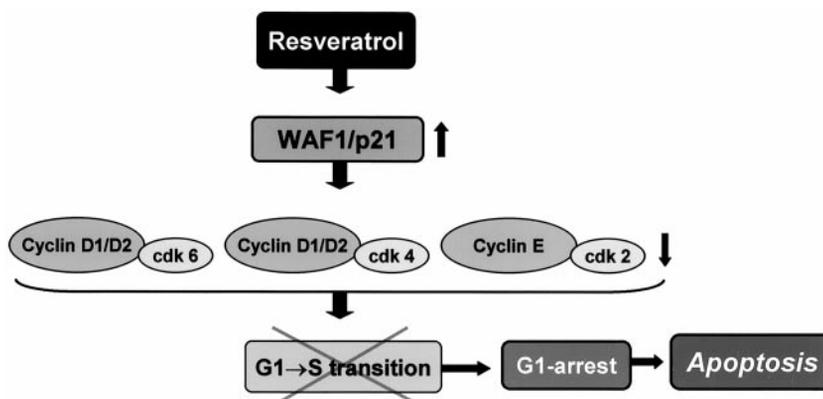
shown the involvement of cell cycle regulation-mediated apo-ptosis as a mechanism of cell growth inhibition (20, 21, 46–50), we investigated the involvement of the cki-cyclin-cdk machin-ery during the induction of cell cycle arrest and apoptosis by resveratrol in A431 cells. It is now well established that in eukaryotes, passage through the cell cycle is governed by the function of a family of protein kinase complexes (25–28). Each complex is composed minimally of a catalytic subunit, the cdk, and its essential activating partner, the cyclin (25–28). Under normal conditions, these complexes are activated at specific intervals and through a series of events and result in the pro-gression of cells through different phases of cell cycle, thereby ensuring normal cell growth (25–28). Any defect in this ma-chinery causes an altered cell cycle regulation that may result in unwanted cellular proliferation ultimately culminating in the development of cancer (25–28). During the progression of the cell cycle, the cdk-cyclin complexes are inhibited via binding to ckis such as the CIP/KIP and INK4 families of proteins (25–28). Because our studies have demonstrated that resveratrol treat-ment of A431 cells resulted in a G_1-phase arrest of the cell cycle, we examined the effect of resveratrol on cell cycle-regulatory molecules operative in the G_1 phase of the cell cycle.

Our data demonstrated a significant up-regulation of the cki WAF1/p21 during G_1-phase arrest and apoptosis of these cells by resveratrol. Many studies have shown that exogenous stimuli may result in a p53-dependent as well as a p53-inde-pendent induction of WAF1/p21, which may cause a blockade of G_1-S-phase transition resulting in a G_1-phase cell cycle arrest and apoptosis (19, 25–28, 45, 46). In the absence of active p53 in A431 cells, the observed induction of WAF1/p21 by resvera-trol appears to be independent of wild-type p53. However, the role of mutant p53 during this process remains to be examined. Because WAF1/p21 is regarded as a universal inhibitor of cyclin-cdk complexes (18, 19, 25–28, 45), we assessed the effect of resveratrol treatment on the cyclins and cdks operative in the G_1 phase of the cell cycle, specifically, cyclins D1, D2, and E and cdk2, cdk4, and cdk6. Resveratrol treatment of the cells was found to result in significant down-modulation of all of these regulatory molecules, although to a different extent. Studies have shown that the ckis inhibit the kinase activities associated with cdk-cyclin complexes, thereby modulating the phosphorylation events, which are believed to play the key role in progression of the cell cycle at many checkpoints (18, 19, 25–28, 45). Therefore, we examined the effect of resveratrol on

kinase activities associated with cyclin-cdk complexes in the immunoprecipitates of cdk2, cdk4, and cdk6 proteins. Resveratrol treatment was found to result in a dose- and time-dependent inhibition in the kinase activities associated with all of the cdks examined.

Our data are in agreement with the fact that the cdks and cyclins operate in association with each other by forming complexes, which are inhibited by ckis (18, 19, 25–28, 45). This series of events impose a blockade of G_1-S-phase transition, thereby causing a G_1-phase arrest of the cell cycle. Based on the outcome of this study and the available knowledge of cell cycle regulation, as shown in the composite scheme in Fig. 7, we suggest the series of events by which resveratrol results in an imposition of an artificial checkpoint at G_1-S-phase transition, thereby resulting in an arrest of cancer cells in the G_1 phase of the cell cycle. This cell cycle arrest, we suggest, is an irreversible process that ultimately results in apoptotic cell death. However, it is also possible that the apoptosis induction by resveratrol may be occurring independently of G_1-phase arrest. Additional studies are needed to clarify this.

Although some studies have shown the regulation of WAF1/p21 by resveratrol (38, 51, 52), to our knowledge, this is the first systematic study showing the involvement of each component of the cki-cyclin-cdk machinery during cell cycle arrest and apoptosis of cancer cells induced by resveratrol.

In recent years, the agents causing cell cycle-mediated apoptosis have gained increasing appreciation as ideal compounds for the management of cancer. Furthermore, in recent years, many synthetic cell cycle inhibitors such as flavopiridol, olomoucine, roscovitine, and puvalanol B have come to be viewed as a new generation of anticancer drugs, and some of these are being investigated for their efficacy against cancer in clinical trials (14, 16, 17, 19, 24, 53). Therefore, naturally occurring agents such as resveratrol could be developed as potent anticancer agents for the management of cancer.

ACKNOWLEDGMENTS

REFERENCES

1. Kelloff, G. J., Crowell, J. A., Steele, V. E., Lubet, R. A., Malone, W. A., Boone, C. W., Kopelovich, L., Hawk, E. T., Lieberman, R., Lawrence, J. A., Ali, I., Viner, J. L., and Sigman, C. C. Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. J. Nutr., 130: 467S–471S, 2000.

2. Hong, W. K., and Sporn, M. B. Recent advances in chemoprevention of cancer. Science (Wash. DC), 278: 1073–1077, 1997.

3. Sporn, M. B., and Suh, N. Chemoprevention of cancer. Carcinogenesis (Lond.), 21: 525–530, 2000.

4. Mukhtar, H., and Ahmad, N. Cancer chemoprevention: future holds in multiple agents. Toxicol. Appl. Pharmacol., 158: 207–210, 1999.

5. Ahmad, N., and Mukhtar, H. Green tea polyphenols and cancer: biologic mechanisms and practical implications. Nutr. Rev., 57: 78–83, 1999.

6. Ray, P. S., Maulik, G., Cordis, G. A., Bertelli, A. A., Bertelli, A., and Das, D. K. The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. Free Radic. Biol. Med., 27: 160–169, 1999.

7. Cadenas, S., and Barja, G. Resveratrol, melatonin, vitamin E, and PBN protect against renal oxidative DNA damage induced by the kidney carcinogen $KBrO_3$. Free Radic. Biol. Med., 26: 1531–1537, 1999.

8. Martinez, J., and Moreno, J. J. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem. Pharmacol., 59: 865–870, 2000.

9. Das, D. K., Sato, M., Ray, P. S., Maulik, G., Engelman, R. M., Bertelli, A. A., and Bertelli, A. Cardioprotection of red wine: role of polyphenolic antioxidants. Drugs Exp. Clin. Res., 25: 115–120, 1999.

10. Clement, M. V., Hirpara, J. L., Chawdhury, S. H., and Pervaiz, S. Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood, 92: 996–1002, 1998.

11. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science (Wash. DC), 275: 218–220, 1997.

12. Surh, Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat. Res., 428: 305–327, 1999.

13. Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J., and Argiles, J. M. Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. Biochem. Biophys. Res. Commun., 254: 739–743, 1999.

14. Buolamwini, J. K. Cell cycle molecular targets in novel anticancer drug discovery. Curr. Pharm. Des., 6: 379–392, 2000.

15. Collins, K., Jacks, T., and Pavletich, N. P. The cell cycle and cancer. Proc. Natl. Acad. Sci. USA, 94: 2776–2778, 1997.

16. Hajduch, M., Havlieek, L., Vesely, J., Novotny, R., Mihal, V., and Strnad, M. Synthetic cyclin dependent kinase inhibitors. New generation of potent anti-cancer drugs. Adv. Exp. Med. Biol., 457: 341–353, 1999.

17. McDonald, E. R., and El Deiry, W. S. Cell cycle control as a basis for cancer drug development. Int. J. Oncol., 16: 871–886, 2000.

18. Sherr, C. J. Cancer cell cycles. Science (Wash. DC), 274: 1672–1677, 1996.

19. Sherr, C. J., and Roberts, J. M. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev., 13: 1501–1512, 1999.

20. Ahmad, N., Feyes, D. K., Nieminen, A. L., Agarwal, R., and Mukhtar, H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J. Natl. Cancer Inst., 89: 1881–1886, 1997.

21. Ahmad, N., Gupta, S., and Mukhtar, H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor κB in cancer cells versus normal cells. Arch. Biochem. Biophys., 376: 338–346, 2000.

22. Deigner, H. P., and Kinscherf, R. Modulating apoptosis: current applications and prospects for future drug development. Curr. Med. Chem., 6: 399–414, 1999.

23. Evan, G., and Littlewood, T. A matter of life and cell death. Science (Wash. DC), 281: 1317–1322, 1998.

24. Stadler, W. M., Vogelzang, N. J., Amato, R., Sosman, J., Taber, D., Liebowitz, D., and Vokes, E. E. Flavopiridol, a novel cyclin-dependent kinase inhibitor, in metastatic renal cancer: a University of Chicago Phase II Consortium study. J. Clin. Oncol., 18: 371–375, 2000.

25. Jacks, T., and Weinberg, R. A. Cell-cycle control and its watchman. Nature (Lond.), 381: 643–644, 1996.

26. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev., 9: 935–944, 1995.

27. Sherr, C. J. G_1 phase progression: cycling on cue. Cell, 79: 551–555, 1994.

28. Sherr, C. J., and Roberts, J. M. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev., 9: 1149–1163, 1995.

29. Mowat, M. R. p53 in tumor progression: life, death, and everything. Adv. Cancer Res., *74:* 25–48, 1998.

30. Gamet-Payrastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M. A., Chevolleau, S., Gasc, N., Tulliez, J., and Terce, F. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res., *60:* 1426–1433, 2000.

31. Xia, W., Spector, S., Hardy, L., Zhao, S., Saluk, A., Alemane, L., and Spector, N. L. Tumor selective G_2/M cell cycle arrest and apoptosis of epithelial and hematological malignancies by BBL22, a benzazepine. Proc. Natl. Acad. Sci. USA, *97:* 7494–7499, 2000.

32. Park, W. H., Seol, J. G., Kim, E. S., Hyun, J. M., Jung, C. W., Lee, C. C., Kim, B. K., and Lee, Y. Y. Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. Cancer Res., *60:* 3065–3071, 2000.

33. Jang, M., and Pezzuto, J. M. Effects of resveratrol on 12-*O*-tetradecanoylphorbol-13-acetate-induced oxidative events and gene expression in mouse skin. Cancer Lett., *134:* 81–89, 1998.

34. Turner, R. T., Evans, G. L., Zhang, M., Maran, A., and Sibonga, J. D. Is resveratrol an estrogen agonist in growing rats? Endocrinology, *140:* 50–54, 1999.

35. Holmes-McNary, M., and Baldwin, A. S. Chemopreventive properties of *trans*-resveratrol are associated with inhibition of activation of the IκB kinase. Cancer Res., *60:* 3477–3483, 2000.

36. Hsieh, T. C., and Wu, J. M. Grape-derived chemopreventive agent resveratrol decreases prostate-specific antigen (PSA) expression in LNCaP cells by an androgen receptor (AR)-independent mechanism. Anticancer Res., *20:* 225–228, 2000.

37. Gautam, S. C., Xu, Y. X., Dumaguin, M., Janakiraman, N., and Chapman, R. A. Resveratrol selectively inhibits leukemia cells: a prospective agent for *ex vivo* bone marrow purging. Bone Marrow Transplant., *25:* 639–645, 2000.

38. Mitchell, S. H., Zhu, W., and Young, C. Y. Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Cancer Res., *59:* 5892–5895, 1999.

39. Surh, Y. J., Hurh, Y. J., Kang, J. Y., Lee, E., Kong, G., and Lee, S. J. Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett., *140:* 1–10, 1999.

40. Hsieh, T. C., Burfeind, P., Laud, K., Backer, J. M., Traganos, F., Darzynkiewicz, Z., and Wu, J. M. Cell cycle effects and control of gene expression by resveratrol in human breast carcinoma cell lines with different metastatic potentials. Int. J. Oncol., *15:* 245–252, 1999.

41. Hsieh, T. C., and Wu, J. M. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. Exp. Cell Res., *249:* 109–115, 1999.

42. Bushman, J. L. Green tea and cancer in humans: a review of the literature. Nutr. Cancer, *31:* 151–159, 1998.

43. Kwok, T. T., Mok, C. H., and Menton-Brennan, L. Up-regulation of a mutant form of p53 by doxorubicin in human squamous carcinoma cells. Cancer Res., *54:* 2834–2836, 1994.

44. Kaelin, W. G. The emerging p53 gene family. J. Natl. Cancer Inst. (Bethesda), *91:* 594–598, 1999.

45. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. The p53 network. J. Biol. Chem., *273:* 1–4, 1998.

46. Ahmad, N., Feyes, D. K., Agarwal, R., and Mukhtar, H. Photodynamic therapy results in induction of WAF1/CIP1/P21 leading to cell cycle arrest and apoptosis. Proc. Natl. Acad. Sci. USA, *95:* 6977–6982, 1998.

47. Ahmad, N., Gupta, S., and Mukhtar, H. Involvement of retinoblastoma (Rb) and E2F transcription factors during photodynamic therapy of human epidermoid carcinoma cells A431. Oncogene, *18:* 1891–1896, 1999.

48. Aranha, O., Wood, D. P., and Sarkar, F. H. Ciprofloxacin mediated cell growth inhibition, S/G_2-M cell cycle arrest, and apoptosis in a human transitional cell carcinoma of the bladder cell line. Clin. Cancer Res., *6:* 891–900, 2000.

49. Sugikawa, E., Hosoi, T., Yazaki, N., Gamanuma, M., Nakanishi, N., and Ohashi, M. Mutant p53 mediated induction of cell cycle arrest and apoptosis at G_1 phase by 9-hydroxyellipticine. Anticancer Res., *19:* 3099–3108, 1999.

50. Algeciras-Schimnich, A., Griffith, T. S., Lynch, D. H., and Paya, C. V. Cell cycle-dependent regulation of FLIP levels and susceptibility to Fas-mediated apoptosis. J. Immunol., *162:* 5205–5211, 1999.

51. Hsieh, T. C., Juan, G., Darzynkiewicz, Z., and Wu, J. M. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21$^{WAF1/CIP1}$, and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G_2. Cancer Res., *59:* 2596–2601, 1999.

52. Tessitore, L., Davit, A., Sarotto, I., and Caderni, G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21^{CIP} expression. Carcinogenesis (Lond.), *21:* 1619–1622, 2000.

53. Senderowicz, A. M., Headlee, D., Stinson, S. F., Lush, R. M., Kalil, N., Villalba, L., Hill, K., Steinberg, S. M., Figg, W. D., Tompkins, A., Arbuck, S. G., and Sausville, E. A. Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasms. J. Clin. Oncol., *16:* 2986–2999, 1998.