[CANCER RESEARCH 61, 6137–6144, August 15, 2001]

Resveratrol Exhibits Cytostatic and Antiestrogenic Properties with Human Endometrial Adenocarcinoma (Ishikawa) Cells[1]

Krishna P. L. Bhat and John M. Pezzuto[2]

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

ABSTRACT

***Trans*-3,4′,5-trihydroxystilbene (resveratrol), a polyphenolic compound found in the human diet, was reported recently to serve as an estrogen agonist with cultured MCF-7 cells transfected with estrogen response element-luciferase reporter plasmids. As currently shown, treatment of cultured human endometrial adenocarcinoma (Ishikawa) cells with resveratrol (concentrations as high as 10 μM) did not significantly increase the levels of an estrogen-inducible marker enzyme, alkaline phosphatase. To the contrary, when alkaline phosphatase was induced by treatment with 1 nM of 17β-estradiol (E_2), resveratrol exhibited a dose-dependent decrease in activity (IC_{50} = 2.3 μM). Furthermore, when Ishikawa cells were treated with resveratrol as a single agent, estrogen-inducible progesterone receptor (PR) was not enhanced, and PR expression induced by treatment with E_2 was inhibited by resveratrol in a dose-dependent fashion at both the mRNA and protein levels. In addition, resveratrol mediated suppression of a functional activity of PR as demonstrated by down-regulation of α_1-integrin expression induced by E_2 plus progesterone. With transient transfection experiments conducted with Ishikawa cells, antiestrogenic effects were confirmed by dose-dependent inhibition of E_2-induced estrogen response element-luciferase transcriptional activity. Because resveratrol antagonized estrogenic effects in Ishikawa cells, competitive binding analyses were performed to examine the potential of displacing [^{3}H]E_2 from human estrogen receptor (ER). Resveratrol showed no discernable activity with ER-α, but with ER-β, E_2 was displaced with an IC_{50} of 125 μM. However, mRNA and protein expression of ER-α but not ER-β were suppressed by resveratrol in Ishikawa cells, in the concentration range of 5–15 μM. In addition, in the presence or absence of E_2, resveratrol inhibited Ishikawa cell proliferation in a time-dependent manner with cells accumulating in the S phase of the cycle ≤48 h. This effect was reversible. Analysis of some critical cell cycle proteins revealed a specific increase in expression of cyclins A and E but a decrease in cyclin-dependent kinase 2. These data suggest resveratrol exerts an antiproliferative effect in Ishikawa cells, and the effect may be mediated by both estrogen-dependent and -independent mechanisms.**

INTRODUCTION

Resveratrol,[3] a constituent of grapes and grape products such as wine, is a phytoalexin generated in response to fungal infection (1). This agent has been postulated to reduce risk of coronary heart disease in certain populations (2). More recent data have suggested cancer chemopreventive effects in various stages of carcinogenesis (3), and a substantial amount of work has been performed to characterize this activity. For example, pretreatment of mouse skin with resveratrol negated several 12-*O*-tetradecanoylphorbol-13-acetate-induced effects including expression of *c-fos* and *transforming growth factor-β1* (4). Similarly, resveratrol has been shown to be a potent inhibitor of cyclooxygenase-2 transcription in mammary epithelial cells (5) and inducible NO synthase in macrophages (6). A recent study demonstrated that resveratrol is a potent inhibitor of inhibitory κB kinase activity and, thus, inhibits nuclear factor kappaB activation and nuclear factor kappaB-dependent gene expression (7). Resveratrol also has been shown to inhibit aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and mRNA expression (8). In addition, the compound possesses antioxidant activity, inhibits protein kinase C (9, 10), and signals apoptosis in human cancer cells (11, 12). It has been speculated that high amounts of resveratrol in red wine may act as an antioxidant, promote nitric oxide production, inhibit platelet aggregation, and increase high-density lipoprotein cholesterol, all contributing to its cardioprotective properties (13).

Phytoestrogens are dietary nonsteroidal compounds that have been speculated to offer protection against estrogen-dependent cancers or heart disease. Most phytoestrogens bind to ERs with much lower affinity than E_2[4] (14) and are weakly estrogenic (15, 16). The decreased incidence of breast cancer in Asian women who consume a high soy diet containing a significant amount of phytoestrogens (17) has been associated with phytoestrogens capable of antagonizing the effects of E_2 (18). However, to date there is no conclusive evidence that phytoestrogens are antiestrogenic. Another possibility is that phytoestrogens exert antiproliferative and cancer-protective effects through an ER-independent pathway (19, 20).

At the present time it is unclear if resveratrol functions as an estrogen agonist or antagonist. Resveratrol has been referred to as a phytoestrogen, exhibiting estrogen agonism and even superagonism (when combined with E_2) with transfected MCF-7 breast cancer cells in culture (21). Others have categorized resveratrol as an antiestrogen with the same cell line at a concentration of ≥1 μM in the presence of E_2 (22). Resveratrol has also been shown to have a direct antiproliferative effect on breast cancer cells, irrespective of their ER status (23). Whereas an estrogenic effect may be viewed as potentially beneficial for cardiovascular response (21), the potential of adverse effects on the endometrium, for example, would be of great concern (24). As currently reported, resveratrol does not mediate estrogenic effects with cultured Ishikawa cells. To the contrary, when tested in combination with E_2, antagonistic effects were observed with resveratrol.

MATERIALS AND METHODS

Chemicals. All of the chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise specified. Resveratrol was supplied by Pharmascience, Montreal, Canada. For all of the experiments, resveratrol and E_2 were dissolved in DMSO as 1000 × stock solutions.

AP Induction in Ishikawa Cells. Ishikawa cells were routinely cultured in DMEM/F-12 medium supplemented with 2 mM glutaMAX-1, antibiotic-antifungal (10 units/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B), 1 mM sodium pyruvate, and 10% FBS. All of the

Received 1/15/01; accepted 6/19/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by P01 CA48112 awarded by the National Cancer Institute. Some of these data were presented at the 15th Asia Pacific Cancer Conference, Chennai, India, December 12–15, 1999.

[2] To whom requests for reprints should be addressed, at the Program for Collaborative Research in the Pharmaceutical Sciences (M/C 877), College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612. Phone: (312) 996-5967; Fax: (312) 996-2815; E-mail: jpezzuto@uic.edu.

[3] The abbreviations used are: resveratrol, *trans*-3,4′,5-trihydroxystilbene; E_2, 17β-estradiol; FBS, fetal bovine serum; ER, estrogen receptor; Ishikawa, human endometrial adenocarcinoma; AP, alkaline phosphatase; RT-PCR, reverse transcription-PCR; PR, progesterone receptor; cdk, cyclin-dependent kinase; GADPH, glyceraldehyde 3-phosphate dehydrogenase; ERE, estrogen response element.

[4] K. P. L. Bhat, D. Lantvit, K. Christov, R. G. Mehta, R. C. Moon, and J. M. Pezzuto. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models, submitted for publication.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

medium components and reagents were purchased from Life Technologies, Inc., Grand Island, NY, except FBS, which was purchased from Atlanta Biologicals, Atlanta, GA. The standard assay has been described in detail elsewhere (25). Briefly, a day before plating the cells, the medium was changed to a phenol red-free formulation of DMEM/F-12 containing charcoal/dextran-stripped FBS to remove estrogens (estrogen-free medium). Cell suspensions (190 μl containing 5×10^4 cells) were plated into 96-well microtiter plates and incubated overnight at 37°C in a 5% CO_2 incubator. Resveratrol (10 μl dissolved in DMSO, diluted 10-fold in ethanol, and then diluted an additional 10-fold in complete phenol red-free medium to reduce any inductive effects of DMSO and ethanol), either alone or with 2×10^{-6} M E_2, and the relevant controls (DMSO, E_2, and tamoxifen) were added to the plated cells and incubated for 4 days. The plates were processed by removing the test medium, washing twice with PBS, adding 50 μl of 0.1% Triton X-100 (v/v) in 0.1 M Tris-HCl buffer (pH 9.8), and freezing at −80°C. For analysis, the plates were rapidly thawed at 37°C, and 150 μl of 0.1 M Tris-HCl buffer (pH 9.8) containing 1 mg/ml of *p*-nitrophenyl phosphate were added to each well. The plates were monitored at 405 nm with an ELISA reader every 15 s with a 10 s shake between each reading for the first 8 min. The slopes of the obtained curves were calculated, and those obtained with cell preparations treated with test samples were compared with standards. The percentage of induction for determination of estrogenic activity was calculated as follows: % induction = ($Slope_{sample} - Slope_{cells}/Slope_{DMSO} - Slope_{cells}$) × 100. For determination of antiestrogenic activity, induction was calculated as ($Slope_{sample} - Slope_{DMSO}/Slope_{estradiol} - Slope_{DMSO}$). Dose-response curves were plotted, and IC_{50} values were calculated.

Analysis of mRNA Expression by Semiquantitative RT-PCR. Ishikawa cells were cultured as described above. Cells (50×10^4 cells/dish) were plated in 100 mm^2 Petri dishes, and compounds were added 24 h later. After 48 h of incubation, RNA was extracted from the cells using TRIzol reagent (Life Technologies, Inc.) followed by isopropanol precipitation. The RNA concentration was determined by measuring absorbance at 260 nm. Total RNA (2 μg) was converted to cDNA using the Superscript preamplification system (Life Technologies, Inc.). Briefly, a mixture of 1 × PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl] containing 2.5 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphate, 0.01 M DTT, 0.5 μg oligo$(dT)_{12-18}$, and 200 units of SuperScript II reverse transcriptase in a 20-μl reaction volume was incubated at 42°C for 50 min. The reverse-transcribed cDNA was diluted to 100 μl with Tris-EDTA buffer [(10 mM Tris-HCl (pH 8.0), 1 mM EDTA)] and 2 μl of the cDNA was amplified in a final volume of 50 μl containing 5 μl of 10 × cDNA PCR buffer [499 mM Tricin-KOH (pH 9.2), 150 mM KOAc, 35 mM $Mg(OAc)_2$, and 750 μg/ml BSA], 1 μl of 10-mM dNTP, 1 μl each of 10-μM sense and antisense primers, and 1 μl of 50 × Advantage II cDNA Polymerase Mix (Clontech, Palo Alto, CA). The primer sequences for PR, ER-α, -β, and GADPH are listed in Table 1. The primer sequences for PR mRNA were adapted from Knowlden *et al.* (26). The PCR reaction was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Perkin-Elmer Corp., Norwalk, CT) with the following parameters: (*a*) a first denaturation step at 94°C for 4 min; followed by (*b*) three temperature cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; followed by (*c*) a complete extension step for 10 min at 72°C. The number of cycles was optimized at 31 after examining a range of 10–40 cycles. The sequences for *ER-α* and *-β* mRNA were adapted from Leygue *et al.* (27). For the analysis of *ER-α* and *-β* mRNA expression, the cycles used were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The first denaturation and final extension steps were kept constant. A cycle number of 35 was chosen as optimal for both *ER-α* and *-β*. After PCR, 10 μl of each PCR product was subsequently separated by gel electrophoresis using 1% agarose (Bio-Rad, Hercules, CA) and visualized by ethidium bromide staining. The primers for *PR* yielded an amplified product of 320 bp. *GADPH* was used as an internal control (336-bp product). The *ER-α* and *-β* primer transcripts yielded DNA of 148 and 222 bp, respectively.

Table 1 *Primers used for RT-PCR analyses of mRNA expression in Ishikawa cells*

mRNA	Primer sequences (5′ → 3′)[a]	Product size (bp)
PR	CCATGTGGCAGATCCCACAGGAGTT TGGAAATTCAACACTCAGTGCCCGG	320
ER-α	TGTGCAATGACTATGCTTCA GCTCTTCCTCCTGTTTTTA	148
ER-β	GTCCATCGCCAGTTATCACATC GCCTTACATCCTTCACACGA	222
GADPH	GGATTTGGTCGTATTGGGCG CTCGGTTTTCCCAGTAGTAGA	336

[a] Primer sets for *PR*, *ER-α*, and *ER-β* were obtained from Life Technologies, Inc. (Grand Island, NY); primer sets for *GADPH* were obtained from Ana-Gen Technologies (Palo Alto, CA).

Western Blot Analysis for Protein Expression. Ishikawa cells were cultured as described above. Cells (50×10^4 cells/dish) were plated in 100 mm^2 Petri dishes, and compounds were added 24 h later. After 48 h of incubation, the cells were lysed using a 2 × electrophoresis sample buffer [250 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol]. For studies involving cell cycle proteins (cdks, cyclins, and cdk inhibitors), cells were exposed to resveratrol for 24 h. Twenty μg of protein lysate were analyzed using SDS-PAGE on a 7.5% polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes by electroblotting and incubated with primary antibody for 2 h. Secondary antibody (antimouse or antirabbit) conjugated to horseradish peroxidase and streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Piscataway, NJ) were diluted 1:2000 and incubated with the membrane for 30 min. Proteins were detected using an enhanced chemiluminescence plus detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech). Bands corresponding to the amount of protein expressed on X-ray films were quantified using Schion imaging software. Anti-PR mouse monoclonal antibody (Ab-8) was purchased from Lab Vision Corporation (Fremont, CA). Anti-ER-α (*sc*-542) and -β (*sc*-8974) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cdk1/cdc2, anti-cdk2, anti-cdk4, anti-cyclin A, anti-$p21^{WAF1}$, and anti-$p27^{KIP1}$ mouse monoclonal antibodies were purchased from BD Transduction Laboratories (San Diego, CA). Anti-cyclin D_1, anti-cyclin D_2, and anti-cyclin E mouse monoclonal antibodies were purchased from BD PharMingen Laboratories (San Diego, CA). Anti-β-actin mouse monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO).

Immunofluoresence for PR and α1 Integrin Expression. For immunohistochemical studies with Ishikawa cells, the cells were maintained as described above. At 70% confluency, the medium was changed to the phenol red-free medium, and cells were trypsinized and grown on 4-well Lab Tek II chamber slides (Nalge Nunc International, Naperville, IL) at a concentration of 1×10^4 cells/well. Twenty-four h after plating, the medium was changed to medium containing resveratrol with or without E_2 (10^{-9} M), and after 48 h, the cells were fixed in 10% buffered-formalin. To block nonspecific antibody reactions, cells were treated with 5% dried skim milk for 10 min and then incubated with primary antibody against PR (Lab Vision Corporation, Fremont, CA) or α1 integrin (Santa Cruz Biotechnology) for 3 h. The tissues were rinsed in PBS and incubated with FITC-conjugated goat antimouse secondary antibody (Santa Cruz Biotechnology) in a dark chamber for 45 min. The slides were mounted using Vectashield mounting medium containing DAPI-2HCl′ (Vector Labs, Burlingame, CA) as counter stain and observed using confocal microscopy.

Transfection and Luciferase Assays. The plasmid (pUC 18) containing the ERE-luciferase construct consisted of two copies of *Xenopus* vitellogenein A_2 ERE (GGTCACAGTGACC) inserted upstream (−331 to −289) of a minimal thymidine kinase promoter (−109 to +45) linked to the luciferase gene. For transfection assays, cells were grown in estrogen-free medium for 24 h and then plated in 12-well plates at a density of 10×10^4 cells/well. The experimental medium containing various concentrations of resveratrol and E_2 were added 24 h later. Two h after changing to experimental medium, the cells were transfected with the lipid-plasmid complex using FUGene 6 reagent (Roche Biochemicals, Indianapolis, IN). Luciferase activity was detected ~24 h after transfection using a Luciferase Assay System (Promega Corporation, Madison, WI) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

ER-α and -β Binding. Pure human recombinant ER-α (specific content 5000 pmol/mg; 0.44 mg/ml protein) and -β (specific content 6098 pmol/mg; 0.36 mg/ml protein) were obtained from PanVera Corporation, Madison, WI, and [2,4,6,7-^{3}H]E_2 was purchased from NEN Life Science Products, Inc., Boston, MA. The procedure of Obourn *et al.* (28) was slightly modified. Twenty-four h before the assay, a 50% v/v hydroxylapatite slurry was prepared using 10 g of hydroxylapatite/60 ml of TE buffer [50 mM Tris-HCl (pH 7.4) and 1 mM EDTA] and stored at 4°C. ER binding buffer [10 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, and 1 mg/ml BSA], ER-α [40 mM

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

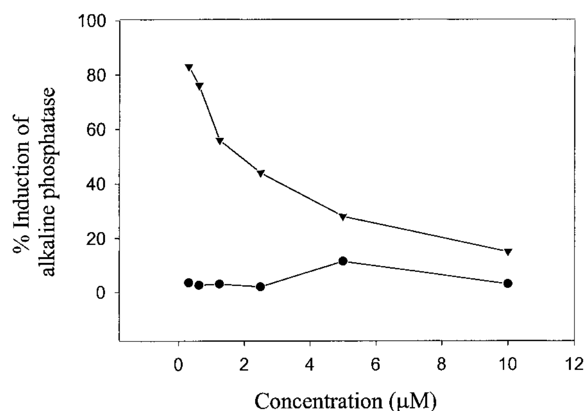

Fig. 1. Effect of resveratrol on induction of AP in Ishikawa cells. Cells were treated with various concentrations of resveratrol with (▼) or without (●) E_2 (1 nM) for 96 h in estrogen-free medium, and AP levels were measured using *p*-nitrophenyl phosphate substrate as described in "Materials and Methods." Data points represent mean of triplicate measurements. SE *bars* are too small to be viewed with the data points.

Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, and 1 mM EGTA] and -β [40 mM Tris-HCl (pH 7.5)] wash buffers were prepared subsequently. The reaction mixture consisted of 5 μl of a 1:20 diluted ER sample in ER binding buffer, 5 μl of 'Hot Mix' {prepared fresh using 3.2 μl of [^{3}H]E_2 (25 μM; 83 Ci/mmol), 98.4 μl of ethanol, and 98.4 μl of ER binding buffer}, and 90 μl of ER binding buffer. For inhibition studies, various concentrations of resveratrol and E_2 (in DMSO) ranging from 0.5 nM to 50 μM were included in the mixture. The incubations were performed at room temperature for 2 h after which 100 μl of 50% hydroxylapatite slurry were added, and the tubes were incubated on ice for 15 min with vortexing every 5 min. Respective ER wash buffers were added (1 ml), and the tubes were vortexed and centrifuged at 2000 × *g* for 5 min. The supernatant was discarded, and this wash step was repeated 3 times. The hydroxylapatite pellet that contained bound E_2 was then resuspended in 200 μl of ethanol, transferred to scintillation vials, 4 ml of Cytoscint (ICN, Costa Mesa, CA) were added, and the vials were counted by liquid scintillation. All of the assays performed had blank reaction mixtures without the receptor and solvent control reaction mixtures. The percentage of [^{3}H]E_2 binding to the ER was determined as: $(dpm_{sample} - dpm_{blank}/dpm_{DMSO} - dpm_{blank}) - 1 \times 100$. Dose-response curves were plotted, and IC_{50} values were calculated.

Cell Growth and Colony Formation Assays. Ishikawa cells were routinely maintained as described above. Twenty-four h before plating, the medium was changed to phenol red-free medium. High density cultures were exposed to a trypsin (0.05%)-EDTA (0.02%) solution for 10–15 min at 37°C, and the collected cells were seeded into 100-mm^2 dishes at a density of 10×10^4 cells/dish. Three days later, the medium was changed to experimental medium containing either DMSO (solvent control), E_2 (1 nM), resveratrol (10 μM), or resveratrol plus E_2. For growth experiments, the cells were harvested by trypsin-EDTA treatment and counted in a Neubauer-Levy hemocytometer under an inverted microscope. For colony formation experiments, the cells were cultured as described above. Cells in the log phase of growth were plated on 100-mm tissue culture dishes (500 cells/dish) and treated with 20 μM of resveratrol after a 24-h preincubation period. After an additional 12, 24, 36, or 48 h, the cells were washed with PBS and cultured in resveratrol-free medium for 1 week. Cell colonies were then fixed with methanol, stained with Giemsa (Fisher Scientific), and counted using a colony counter (Mini count; Imaging Products International Inc., Chantilly, VA). The percentages of cells surviving relative to solvent-treated controls were calculated.

Flow Cytometry. Flow cytometric analyses were performed according to the method of Vindelov *et al.* (29). Cells were plated in 100-mm^2 dishes at a concentration of 20×10^4 cells/Petri dish. Medium containing relevant test agents were added 24 h after changing to estrogen-free medium. After an additional 12, 24, 36, or 48 h, the medium were removed and cells were detached using trypsin-EDTA and fixed using 70% aqueous ethanol. Cell pellets were obtained by centrifugation and resuspended in 200 μl of citrate buffer [250 mM sucrose, 40 mM trisodium citrate, and 0.05% v/v DMSO (pH 7.6)] and 1.8 ml of 3 mg of trypsin/100 ml of stock buffer [3.4 mM trisodium citrate, 0.1% v/v NP40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris-HCl (pH 7.6)]. The suspension was incubated at room temperature for 10 min after vortexing twice. The cell suspension was then treated with 1.5 ml of trypsin inhibitor (25 mg/100 ml) and RNase A (10 mg/100 ml) in stock buffer and incubated at room temperature for 10 min after vortexing twice. Finally, 2 ml of a mixture of propidium iodide (41.6 mg/100 ml) and spermine tetrahydrochloride (116 mg/100 ml) in stock buffer were added. Cellular DNA content was analyzed by flow cytometry using a Coulter EPICS ELITE ESP laser-based flow cytometer. At least 15,000 cells were used for each analysis.

RESULTS

Resveratrol Suppresses E_2-inducible AP in Ishikawa Cells. Ishikawa cells were grown in phenol red-free DMEM/F-12 medium that contained steroid-stripped FBS for all of the studies. Resveratrol did not significantly induce AP activity when administered alone at any of the concentrations tested (Fig. 1). When cells were treated with 1 nM E_2, a 15-fold increase in AP activity was observed, and resveratrol mediated a marked dose-dependent antiestrogenic effect, as evident from a decrease in the induction of AP activity. The IC_{50} was calculated as 2.3 μM. Resveratrol did not affect cell viability at any of the concentrations tested, as judged by parallel studies in which growth inhibition (25) was assessed using 0.4% sulforhodamine B dye (data not shown).

Resveratrol Down-Regulates *PR* mRNA Expression in Ishikawa Cells. To additionally characterize the antiestrogenic effect of resveratrol, the effect on E_2-inducible *PR* mRNA was examined with Ishikawa cells. After changing to estrogen-free medium for 24 h, the basal level of *PR* expression was minimal. Using an incubation period of 48 h, resveratrol was tested at four different concentrations with or without E_2. As shown in Fig. 2*A*, *PR* was not induced by resveratrol at any of the concentrations tested. When administered with E_2, marked down-regulation of *PR* expression was observed at a concentration of 15 μM to a level comparable with the E_2-free control group. Suppression was also evident at 5 and 10 μM of resveratrol, but at 1 μM, expression was restored to a level comparable with that of the

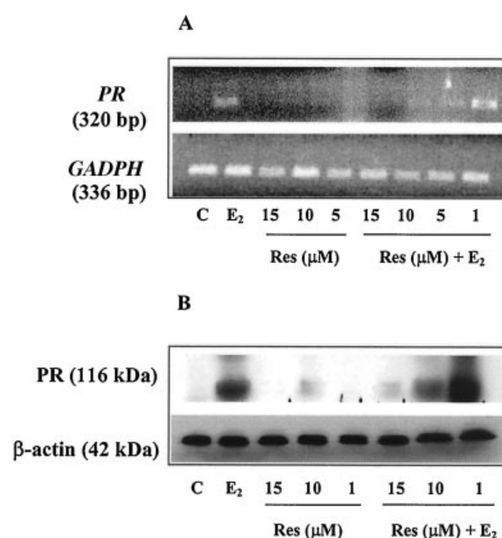

Fig. 2. Effect of resveratrol on PR expression in Ishikawa cells. Cells were treated with various concentrations of resveratrol with or without E_2 (1 nM), and expression of mRNA and protein were analyzed using RT-PCR and Western blotting, respectively, as described in "Materials and Methods." *A*, PCR products of *PR* and *GADPH* in resveratrol-treated Ishikawa cells. In each case, 15 μl of PCR product was loaded onto each well of a 2% agarose gel. *B*, immunoblots were performed using anti-PR mouse antibody, followed by stripping and reprobing with anti-β-actin specific mouse antibody. Immunoreactive bands were identified using enhanced chemiluminescence. Equal sample loading was also confirmed by Ponceau S staining of the Western blot membrane (not shown). *Res,* resveratrol.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

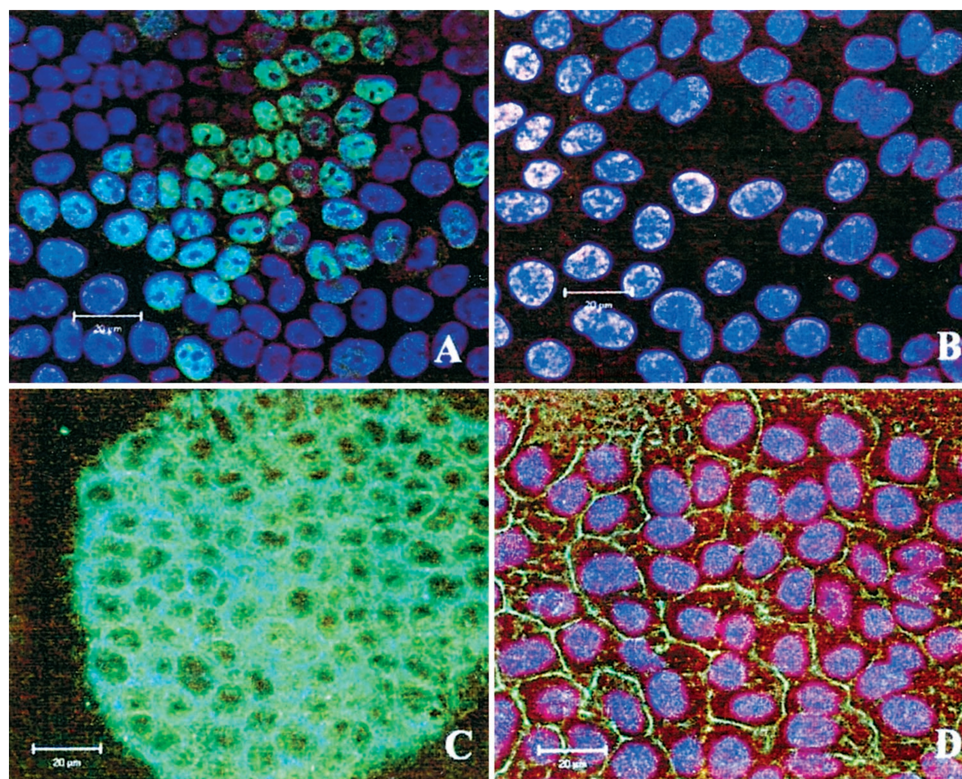

Fig. 3. Effect of resveratrol on immunolocalization of PR and α1 integrin protein in Ishikawa cells. Cells were treated were treated with E_2 (1 nM; *A* and *C*) or E_2 (1 nM) and 20 μM resveratrol (*B* and *D*), fixed in formalin, and incubated with various antibodies as described in "Materials and Methods." Fluorescent images of FITC and 4′-6-diamidino-2-phenylindole-2HCl′ (counterstain) stained cells were then captured using confocal microscopy. *A*, E_2-treated cells expressing PR protein (*green*) localized in the nuclei, compared with *B*, E_2-induced PR is down-regulated by resveratrol. *C*, cells expressing high levels of α1 integrin protein (*green*) localized in the cell membrane on E_2 (1 nM) + progesterone (1 μM) treatment and *D*, corresponding down-regulation in resveratrol-treated cells. Nuclei that did not express PR or α1 integrin were counterstained by DAPI (*blue*) in *A, B,* and *D*. *Bar* = 20 μm.

E_2-treated group. The reverse-transcription and the loading efficiency were monitored by including *GADPH* as an internal control.

Resveratrol Down-Regulates PR and α1 Integrin Protein Expression in Ishikawa Cells. Additional studies were performed to determine the effect of resveratrol on PR protein expression in Ishikawa cells by Western blotting and immunofluorescence. As shown by time course studies, PR stimulation by E_2 (1 nM) was found to be optimal and statistically significant ($P < 0.001$) after an incubation period of 48 h. Consistent with *PR* mRNA expression studies, resveratrol had no effect on basal levels of PR when administered as a single agent. However, when combined with E_2 at a concentration of 15 μM, resveratrol completely inhibited the expression of PR. The reduction was dose-dependent, and PR expression was reduced by ~30% at a resveratrol concentration of 10 μM ($P < 0.005$; Fig. 2*B*). Immunostaining of Ishikawa cells demonstrated that PR was localized predominantly in the nuclei. E_2 (1 nM) significantly enhanced the intensity of staining compared with control (Fig. 3*A*), and resveratrol treatment suppressed this effect when administered with E_2 (Fig. 3*B*). To test the functionality of PR induction and suppression, we examined the expression of α_1 integrin, a collagen-laminin receptor protein that is hormonally regulated in the endometrium. Treatment with E_2 alone did not induce this protein. However, E_2 and progesterone (1 μM) treatment caused induction of α_1 integrin (Fig. 3*C*). When tested at a concentration of 15 μM, resveratrol suppressed this induction (Fig. 3*D*).

Resveratrol Functions as an Antagonist for ER-mediated Luciferase Transcriptional Activation. To additionally characterize the antiestrogenic effects of resveratrol, we performed transient transfection studies in Ishikawa cells. Ligand-activated ER binds to ERE, and its functional activation was tested using a luciferase reporter gene linked to its promoter (ERE). E_2 (1 nM) as a single agent caused a 10-fold increase in luciferase activity compared with control (Fig. 4). Consistent with the results described above, resveratrol did not induce transcriptional activation at any of the concentrations tested (2.5–15 μM). To the contrary, resveratrol exhibited dose-dependent antiestrogenic activity when combined with E_2, as shown in Fig. 4. The effect was maximal at 15 μM, where 100% inhibition of E_2-induced luciferase activity was observed. At 10 μM, resveratrol caused ~70% inhibition.

Characterization of ER Binding by Resveratrol. Because resveratrol antagonized E_2-induced effects in Ishikawa cells, competitive binding studies were performed with ER-α and -β to establish if the response was ER-mediated. As anticipated (Fig. 5, *A* and *B*), unlabeled E_2 reduced the binding of the $[^3H]E_2$ in a dose-dependent fashion, yielding IC_{50} values of 6.4 and 6.2 nM for ER-α and -β, respectively. Resveratrol was tested at concentrations ranging from 0.01–100 μM, and no significant competition for E_2 binding with

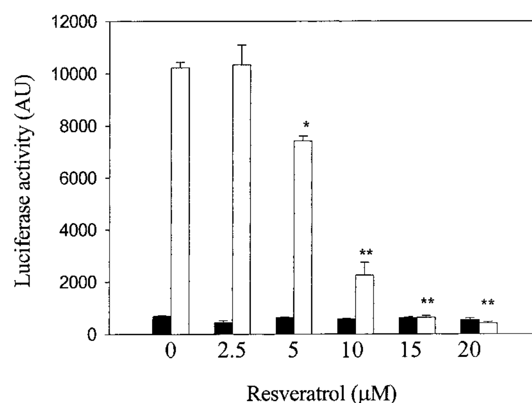

Fig. 4. Effects of resveratrol on ERE-luciferase activity with transient transfected Ishikawa cells. Cells were treated with indicated concentrations of resveratrol alone (■) or in combination with 1 nM E_2 (□) as described in "Materials and Methods." Two h later, cells were transfected with ERE-luciferase plasmid, and luciferase activity was measured after 24 h. Induction values are averaged from triplicate experiments. *Columns*, average of triplicate samples; *bars*, SE. Values with * are significantly different from the respective controls. *, $P < 0.05$; **, $P < 0.005$ (Student's *t* test).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

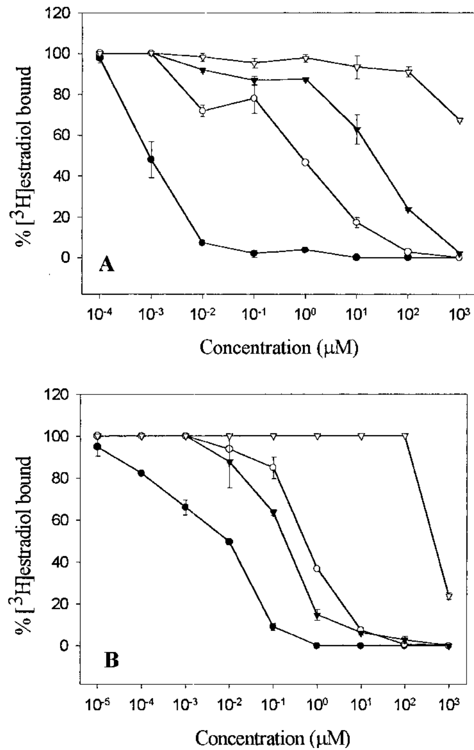

Fig. 5. Competitive inhibition studies of E_2 and resveratrol on binding of [^{3}H]E_2 to ER. Pure recombinant human ER-α (*A*) and -β (*B*) were incubated with [^{3}H]E_2 in the presence or absence of various concentrations of unlabeled E_2 (●), tamoxifen (○), genistein (▼), or resveratrol (▽) for 2 h. Bound ER was trapped using a hydroxylapatite slurry and radioactivity measured by scintillation counting as described in "Materials and Methods." Data points represent mean of the percentage of [^{3}H]E_2 bound using triplicate measurements; *bars,* ± SE.

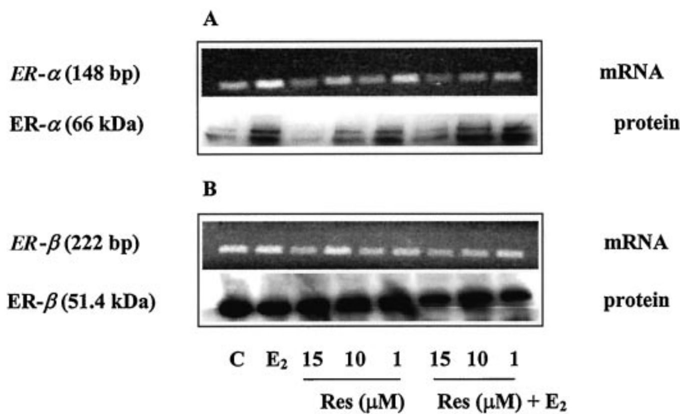

Fig. 6. Effect of resveratrol on ER expression in Ishikawa cells. Cells were treated with various concentrations of resveratrol with or without E_2 (1 nM), and the expression of mRNA and protein were analyzed using RT-PCR and Western blotting, respectively, as described in "Materials and Methods." *A*, PCR products of *ER-α* and -β. After PCR, 15 μl of PCR product were loaded on to each well of a 2% agarose gel. *B*, immunoreactive bands of ER-α and -β. Immunoreactive bands for ER protein were identified using enhanced chemiluminescence. *Res,* resveratrol.

ER-α or -β was observed. Because previous reports (21, 38) have shown binding of resveratrol at concentrations ranging from 10–100 μM, we performed additional dose-response studies with concentrations ranging from 100-1000 μM. Resveratrol preferentially bound to ER-β (IC_{50} = 125 μM) relative to ER-α, where 50% binding inhibition could not be achieved even at a concentration of 1000 μM. Using the same procedures, tamoxifen and genistein were used as positive controls. Consistent with previous results (30), differential competitive binding was observed with genistein. The respective IC_{50} values with ER-α and -β were 29.2 and 0.25 μM. However, tamoxifen displaced the binding of E_2 to ER-α (IC_{50} = 0.12 μM) and -β (IC_{50} = 0.48 μM) with comparable affinity.

Resveratrol Down-Regulates ER-α in Ishikawa Cells. Because the antiestrogenic effects of resveratrol could not be explained by binding to ER, the effect of resveratrol on the expression of ER subtypes was evaluated at the mRNA and protein levels. As shown in Fig. 6, *A* and *B*, estradiol treatment of Ishikawa cells caused an increase in the expression of *ER-α* within 48 h that was significant at the protein level. As a single agent, 15 μM of resveratrol suppressed the basal levels of ER-α (mRNA and protein). No effect was seen at 10 μM, and at 1 μM, an increase in ER-α expression, relative to the control group, was observed. When combined with E_2, complete down-regulation of the ER-α was seen at 15 μM, but milder effects were seen at 10 and 1 μM. Resveratrol did not suppress either the basal or estrogen-induced levels of ER-β at the mRNA or protein levels (Fig. 6, *A* and *B*). Induction of ER-β by estradiol was marginal compared with the induction ER-α.

Resveratrol Exhibits Cytostatic Effects in Ishikawa Cells. Because resveratrol effectively suppressed estrogenic effects at concentrations ≤15 μM, this concentration was used to study potential effects on cell growth. E_2, at a concentration of 1 nM, stimulated the growth of Ishikawa cells compared with the control group as a function of time (Fig. 7). Resveratrol (10 μM) antagonized this effect, and growth suppression was evident even in the absence of E_2. To determine whether resveratrol was functioning by a cytotoxic or cytostatic mechanism, Ishikawa cells were treated for various periods of time with resveratrol and then analyzed for survival (recovery) in drug-free medium. As shown in Table 2, the number of colonies was not reduced significantly compared with control. These data suggest resveratrol functions by a cytostatic mechanism.

Resveratrol Delays S Phase Progression. To determine whether the observed growth inhibition correlated with an effect on the cell cycle, flow cytometric analyses were performed with Ishikawa cells treated with resveratrol at 15 μM. Cells were lysed at various time

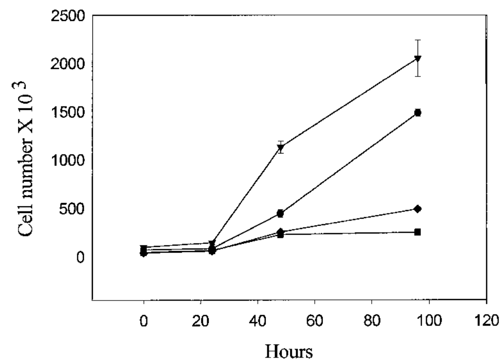

Fig. 7. Effect of resveratrol on growth of Ishikawa cells. Cells, plated at 10 × 10^4 cells/dish, were treated with either an equivolume of DMSO (●), 1 nM E_2 (▼), 10 μM resveratrol with (♦) or without (■) E_2 (1 nM) and counted using a Neubauer-Levy hemocytometer after 24, 48, or 96 h. Values are expressed as mean of triplicate groups; *bars,* ± SE.

Table 2 *Effect of resveratrol on colony formation in Ishikawa cells*[a]

Group	12 h	24 h	36 h	48 h
Control	305 ± 12.7 (100)	320 ± 5.7 (100)	328 ± 4.9 (100)	334 ± 13.4 (100)
Resveratrol (20 μM)	298 ± 7.80 (97.7)	326 ± 6.4 (101.8)	325 ± 7.7 (99)	298 ± 18.4 (89.2)

[a] The values given represent the average number of colonies ± SE (% of control). For experimental details, see "Materials and Methods."

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

points, and nuclei were stained with propidium iodide. Flow cytometric profiles (Fig. 8*A*) reveal an increase in the percentage of S phase cells from ~39% in control to ~68% in resveratrol-treated cells at the end of 24 h. At 36 h, the percentage of cells in S phase was nearly doubled after resveratrol treatment (49%) compared with control (27%). A corresponding decrease in the percentage of cells in the G_1 phase was seen in resveratrol-treated cells. No significant changes in the percentage of cells residing in the G_2 phase were seen. These effects decreased with time, and normal cell cycle dynamics were observed after 48 h. The percentages of cells in different phases of the cell cycle are illustrated as stacked bar graphs in Fig. 8*B*.

Resveratrol Modulates Protein Expression of cdk2 and Cyclins A and E. Because resveratrol caused an increase in cells cycling in the S phase, either an arrest or slowing of the cell cycle machinery in this phase could be possible. An analysis of cell cycle proteins relevant to the G_1-S phases was performed to additionally characterize this effect. Ishikawa cells were treated with 15 μM of resveratrol for 12, 24, 36, and 48 h, and the production of G_1-S cdks, cyclins, and cdk inhibitors was examined by Western blot analyses. The effect of resveratrol on S phase was maximal at 24 h and, hence, the cell cycle proteins were compared at this time point (Fig. 9). The level of cdk2 expression was reduced, but the expression of cyclin A and cyclin E was increased with respect to control. All of the other cyclins and cdks tested were not affected by resveratrol treatment. Also, resveratrol did not affect the expression of cdk inhibitors p21 and p27 (Fig. 9).

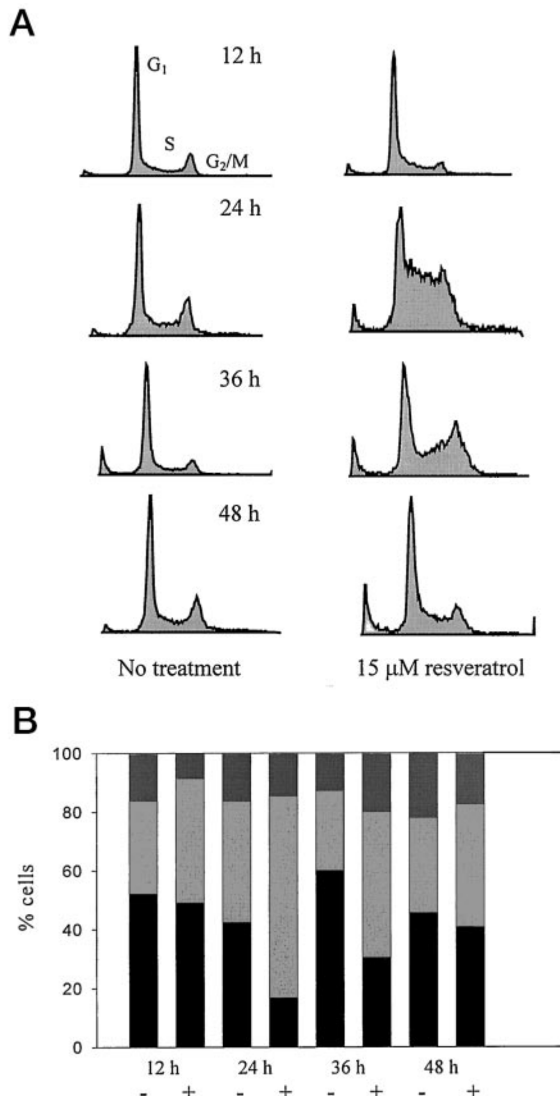

Fig. 8. Effect of resveratrol on cell cycle dynamics in Ishikawa cells. Cells were treated with resveratrol for indicated time periods. *A*, cells were stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry as described in "Materials and Methods." *B*, results from flow cytometry profiles were analyzed for percentage of cells in G_1 (*black*), S (*light gray*), or G_2-M phase (*dark gray*) and illustrated as stacked bar graphs.

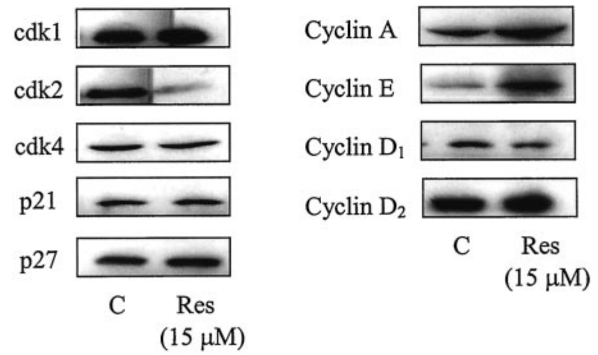

Fig. 9. Changes in cdks, cyclins, and cdk inhibitors. Ishikawa cells were treated with resveratrol (15 μM) for 24 h as described in "Materials and Methods." Relative to DMSO controls, protein expression was compared using Western blot analyses. Immunoreactive bands for the proteins were identified using enhanced chemiluminescence. *C,* control; *Res,* resveratrol.

DISCUSSION

The ostensible cardiovascular benefits of red wine consumption have been attributed to the presence of resveratrol and other antioxidants found in grapes and wine (2). Inhibition of platelet activation may contribute to this effect (2). More recently, resveratrol was characterized as a phytoestrogen with potent estrogen agonist and even superagonist effects in transfected MCF-7 cells (21), and this was provided as an ancillary explanation of cardioprotective action. However, in the same cell line, estrogen antagonist effects have been reported with resveratrol in the presence of E_2 (22), leading to the suggestion of resveratrol serving as a partial agonist in the absence of E_2 and an ER antagonist in the presence of E_2, as indicated by a dose-dependent inhibition of *PR* gene expression. At the mRNA level, resveratrol also suppressed basal levels of *TGF-α* and *IGF-I* expression in MCF-7 cells (22). Others report superestrogenic activity at intermediate concentrations (10 and 25 μM) with transfected MCF-7 cells and antiestrogenic effects at lower concentrations such as 0.1 and 1 μM (31). Estrogenic effects have been attributed to structure similarities between resveratrol and diethylstilbestrol, a synthetic estrogen, and its ability to bind to the ER, but the actual mechanism of action of resveratrol in estrogen-responsive cancer cell lines remains unknown.

Thus, as implied by some of these reports, resveratrol may have beneficial effects, but the suggestion of estrogen superagonist activity raises caution for use as a chemopreventive agent. As examples, endometrial carcinomas in postmenopausal women have been attributed to prolonged exposure to estrogens (32); one drawback of using tamoxifen as a chemopreventive agent for breast cancer is the potential risk of promoting endometrial cancer because of its estrogen agonist nature in the endometrium (24). A useful model for studying responses to steroid hormones in endometrial tissues is the Ishikawa cell line. In this cell line, the expression and activity of the enzyme AP is under strict estrogenic control at the transcriptional level (33). We have exploited this property to develop a suitable assay for identifying antiestrogenic plant extracts (25). E_2 and 4-hydroxy-tamoxifen cause an increase in AP activity in Ishikawa cells (34), and this response can be inhibited by antiestrogens. As currently reported, resveratrol func-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

tioned as an antiestrogen by decreasing E_2-induced AP activity in a dose-dependent manner with an IC_{50} of 2.3 μM. Additionally, when Ishikawa cells were treated with resveratrol as a single agent, AP activity was not induced.

To additionally establish if the down-regulation of AP was an ER-dependent effect, we studied the effect of resveratrol on Ishikawa cells transfected with estrogen-responsive ERE-luciferase. In this model, no estrogenic activity was observed with resveratrol. When combined with E_2 (1 nM), resveratrol antagonized the expression of luciferase in a dose-dependent manner, again suggesting an antiestrogenic effect. However, it may be suggested that transient transfection experiments do not fully recapitulate *in vivo* conditions required to observe agonist activity of antiestrogens, because 4-hydroxy-tamoxifen exhibits estrogen agonist activity in Ishikawa cells stably transfected with reporter constructs containing ERE linked to chloramphenicol acetyl transferase but not in transient transfection assays using Ishikawa cells (35). Thus, to address the reliability of transient transfection assays, we also evaluated the effect of resveratrol on an estrogen-inducible gene. Ishikawa cells contain both a functional ER and E_2-inducible PR, which possesses characteristics similar to PR in normal and neoplastic endometrium (36). In our studies, PR expression was completely absent in control groups when the medium contained steroid-stripped FBS, and the addition of E_2 (1 nM) increased expression, as confirmed by RT-PCR, western blots, and immunofluorescence. At concentrations ranging from 1–15 μM, resveratrol had no effect on PR expression. On the other hand, when resveratrol was combined with E_2, PR expression was decreased at both the mRNA and protein levels. Therefore, consistent with observations obtained with the ERE-luciferase constructs and AP induction, when studying PR, no estrogenic effects were noted with resveratrol, only antiestrogenic effects were observed. In addition, resveratrol down-regulated the expression of the progesterone-responsive collagen-laminin receptor α1 integrin. Although the exact role of integrins is unknown, they have been shown to play a role in biological processes including cell growth, differentiation, migration, and apoptosis (37).

Whereas some researchers have shown that resveratrol binds to ER from MCF-7 cells (IC_{50} ~10 μM) and rat uterine cytosolic extracts (IC_{50} ~100 μM), other studies have shown that resveratrol does not bind to ER in cell extracts from PR1 pituitary cells or partially purified mouse uterine, even at concentrations as high as 100 μM (14, 21, 38, 39). The relative expression of ER-α and -β in these tissue types may account for these differences. Bowers *et al.* (40) have reported that resveratrol binds to purified human ER-α and -β with IC_{50} values of 58.5 and 130 μM, respectively. As currently reported, based on competitive binding analyses, resveratrol did not interact with ER-α or -β at concentrations (1–15 μM) where antiestrogenic activity was seen. At high concentrations (>100 μM), slight affinity for ER-β was observed. The reason for differences between our results and those of Bowers *et al.* (40) is unknown. However, in any case, down-regulation of PR expression and antagonism in transient transfection studies was achieved at resveratrol concentrations much less than those reported for significant binding to ER-α or -β. On the other hand, resveratrol selectively down-regulated ER-α at lower concentrations with Ishikawa cells, and this might be the mechanism through which it acts as an antagonist. Moreover, in recent studies conducted with immature Wistar rats, resveratrol decreased uterine weight and suppressed the expression of ER-α mRNA and protein and *PR* mRNA, similar to antiestrogens (41). However, other studies using ER^- mammary cancer cells have shown that resveratrol has a direct antiproliferative effect that is independent of ER status of the cells (23). In sum, it appears that resveratrol can suppress proliferation by both ER-dependent and -independent mechanisms.

With Ishikawa cells, growth inhibition results from a cytostatic mechanism, as indicated by colony formation assays, and the cells experience a significant prolongation of the S phase with a corresponding decrease in the G_1 phase. Several mechanisms could be responsible for this effect. For example, camptothecin is an S phase-specific DNA-damaging agent (42). Shao *et al.* (43) have shown that treatment of HT29 cells with camptothecin causes a S phase delay with a corresponding increase in cyclin A levels and cdk2 activity. In our studies, resveratrol caused increases in cyclin A and E expression and a corresponding decrease in cdk2 expression. Cyclin A is important for G_1-S transition and progression through S phase, whereas cyclin E is required for G_1-S phase transition. Cdk2 binds to both cyclin A and E and is essential for entry into S phase, and the down-regulation could relate to the mechanism by which resveratrol modulates the cell cycle. However, additional mechanisms may apply that would be worthy of additional investigation. As examples, IFN-β induces S phase slowing (44), apparently through up-regulation of promyelocytic leukemia protein, a matrix-associated nuclear phosphoprotein of which the overexpression induces cell growth inhibition. Also, previous studies have shown that an ornithine decarboxylase inhibitor, difluoromethylornithine, does not affect the G_1-S transition but prolongs S phase in Chinese hamster ovary cells (45). Resveratrol has been shown to inhibit ornithine decarboxylase activity in human colon cancer cells at a concentration of 25 μM (46), and this may have contributed to a similar effect in Ishikawa cells. Furthermore, resveratrol has been reported to inhibit the activity of ribonucleotide reductase and DNA polymerase in some cells, thereby hampering DNA synthesis (47, 48). With Ishikawa cells, the decrease in G_1 phase cells shows that resveratrol may not have an effect in the G_1-S transition. Rather, the S phase machinery may be perturbed.

Similar resveratrol-induced increases in the S phase have been reported with other cell types (49, 50). With a highly invasive mammary cancer cell line, MDA-MB-435, resveratrol (25 μM) caused a similar accumulation in S phase with a corresponding decrease in the G_1 phase; G_2 phase remained unaffected (51). With U937 cells, resveratrol causes a reversible S phase arrest with a corresponding increase in cyclins A and E (52). As demonstrated by the current study, resveratrol causes S phase progression delay but not arrest, with a corresponding decrease in cell growth.

In summary, the data presented here suggest resveratrol mediates antiestrogenic effects in Ishikawa cells by a novel mechanism that involves selective down-regulation of ER-α but not ER-β, manifested as suppression of AP and ERE-luciferase activities, as well as PR and α1 integrin expression. In addition, resveratrol exhibits cytostatic activity in these cells by prolonging S phase and selectively modulating cyclins A and E and cdk2 expression. With mammary cancer cell lines, we have observed recently that resveratrol exhibits mixed estrogenic/antiestrogenic properties in some ER^+ cells (as seen by differential effects on reporter gene assays as well as natural estrogen-responsive genes such as PR), acts as pure antiestrogen in other mammary cells, and, in rodent models, inhibits the formation of carcinogen-induced preneoplastic mammary lesions and mammary tumors (4). Taken together, these data support the notion that resveratrol functions as a novel selective ER modulator. To our knowledge, resveratrol is the first example of a compound to show a tissue-specific profile of this type, and additional studies to characterize the action of resveratrol, as a single agent or in combination with a substance such as tamoxifen, should be of great interest.

ACKNOWLEDGMENTS

We thank Pharmascience, Montreal, Canada for the supply of resveratrol, Dr. Richard B. Hochberg, Yale University, New Haven, CT, for providing

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024

Ishikawa cells, and Dr. Peter J. Kushner, University of California at San Francisco, San Francisco, CA, for providing ERE-luciferase plasmid.

REFERENCES

1. Langcake, P., and Pryce, R. J. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol. Plant Pathol., *9:* 77–86, 1976.
2. Kopp, P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? Eur. J. Endocrinol., *138:* 619–620, 1998.
3. 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.
4. 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.
5. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem., *273:* 21875–21882, 1998.
6. Chun, Y. J., Kim, M. Y., and Guengerich, F. P. Resveratrol is a selective human cytochrome P450 1A1 inhibitor. Biochem. Biophys. Res. Commun., *262:* 20–24, 1999.
7. Holmes-McNary, M., and Baldwin, A. S., Jr. Chemopreventive properties of *trans*-resveratrol are associated with inhibition of activation of the IκB kinase. Cancer Res., *60:* 3477–3483, 2000.
8. 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.
9. Fremont, L., Belguendouz, L., and Delpal, S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. Life Sci., *64:* 2511–2521, 1999.
10. Stewart, J. R., Ward, N. E., Ioannides, C. G., and O'Brian, C. A. Resveratrol preferentially inhibits protein kinase C-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism. Biochemistry, *38:* 13244–13251, 1999.
11. 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.
12. 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.
13. Soleas, G. J., Diamandis, E. P., and Goldberg, D. M. Resveratrol: a molecule whose time has come? and gone? Clin. Biochem., *30:* 91–113, 1997.
14. Stahl, S., Chun, T. Y., and Gray, W. G. Phytoestrogens act as estrogen agonists in an estrogen-responsive pituitary cell line. Toxicol. Appl. Pharmacol., *152:* 41–48, 1998.
15. Sathyamoorthy, N., Wang, T. T., and Phang, J. M. Stimulation of pS2 expression by diet-derived compounds. Cancer Res., *54:* 957–961, 1994.
16. Markiewicz, L., Garey, J., Adlercreutz, H., and Gurpide, E. *In vitro* bioassays of non-steroidal phytoestrogens. J. Steroid Biochem. Mol. Biol., *45:* 399–405, 1993.
17. Messina, M. J., Persky, V., Setchell, K. D., and Barnes, S. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. Nutr. Cancer, *21:* 113–131, 1994.
18. Makela, S. I., Pylkkanen, L. H., Santti, R. S., and Adlercreutz, H. Dietary soybean may be antiestrogenic in male mice. J. Nutr., *125:* 437–445, 1995.
19. Zava, D. T., and Duwe, G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. Nutr. Cancer, *27:* 31–40, 1997.
20. Le Bail, J. C., Champavier, Y., Chulia, A. J., and Habrioux, G. Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci., *66:* 1281–1291, 2000.
21. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc. Natl. Acad. Sci. USA, *94:* 14138–14143, 1997.
22. Lu, R., and Serrero, G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J. Cell. Physiol., *179:* 297–304, 1999.
23. Mgbonyebi, O. P., Russo, J., and Russo, I. H. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. Int. J. Oncol., *12:* 865–869, 1998.
24. Decensi, A., Fontana, V., Bruno, S., Gustavino, C., Gatteschi, B., and Costa, A. Effect of tamoxifen on endometrial proliferation. J. Clin. Oncol., *14:* 434–440, 1996.
25. Pisha, E., and Pezzuto, J. M. Cell-based assay for the determination of estrogenic and anti-estrogenic activities. Methods Cell Sci., *19:* 37–43, 1997.
26. Knowlden, J. M., Gee, J. M., Bryant, S., McClelland, R. A., Manning, D. L., Mansel, R., Ellis, I. O., Blamey, R. W., Robertson, J. F., and Nicholson, R. I. Use of reverse transcription-polymerase chain reaction methodology to detect estrogen-regulated gene expression in small breast cancer specimens. Clin. Cancer Res., *3:* 2165–2172, 1997.
27. Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res., *58:* 3197–3201, 1998.
28. Obourn, J. D., Koszewski, N. J., and Notides, A. C. Hormone- and DNA-binding mechanisms of the recombinant human estrogen receptor. Biochemistry, *32:* 6229–6236, 1993.
29. Vindelov, L. L., Christensen, I. J., and Nissen, N. I. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry, *3:* 323–327, 1983.
30. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology, *139:* 4252–4263, 1998.
31. Basly, J. P., Marre-Fournier, F., Le Bail, J. C., Habrioux, G., and Chulia, A. J. Estrogenic/antiestrogenic and scavenging properties of (E)- and (Z)-resveratrol. Life Sci., *66:* 769–777, 2000.
32. Antunes, C. M., Strolley, P. D., Rosenshein, N. B., Davies, J. L., Tonascia, J. A., Brown, C., Burnett, L., Rutledge, A., Pokempner, M., and Garcia, R. Endometrial cancer and estrogen use. Report of a large case-control study. N. Engl. J. Med., *300:* 9–13, 1979.
33. Albert, J. L., Sundstrom, S. A., and Lyttle, C. R. Estrogen regulation of placental alkaline phosphatase gene expression in a human endometrial adenocarcinoma cell line. Cancer Res., *50:* 3306–3310, 1990.
34. Simard, J., Sanchez, R., Poirier, D., Gauthier, S., Singh, S. M., Merand, Y., Belanger, A., Labrie, C., and Labrie, F. Blockade of the stimulatory effect of estrogens, OH-tamoxifen, OH- toremifene, droloxifene, and raloxifene on alkaline phosphatase activity by the antiestrogen EM-800 in human endometrial adenocarcinoma Ishikawa cells. Cancer Res., *57:* 3494–3497, 1997.
35. Barsalou, A., Gao, W., Anghel, S. I., Carriere, J., and Madler, S. Estrogen response elements can mediate agonist activity of anti-estrogens in human endometrial Ishikawa cells. J. Biol. Chem., *273:* 17138–17146, 1998.
36. Lessey, B. A., Ilesanmi, A. O., Castelbaum, A. J., Yuan, L., Somkuti, S. G., Chwalisz, K., and Satyaswaroop, P. G. Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the $\alpha 1$ integrin. J. Steroid Biochem. Mol. Biol., *59:* 31–39, 1996.
37. Sheppard, D. Epithelial integrins. Bioessays, *18:* 655–660, 1996.
38. Ashby, J., Tinwell, H., Pennie, W., Brooks, A. N., Lefevere, P. A., Beresford, N., and Sumpter, J. P. Partial and weak oestrogenecity of the red wine constituent resveratrol: consideration of its superagonist activity in MCF-7 cells and its suggested cardiovascular protective effects. J. Appl. Toxicol., *19:* 39–45, 1999.
39. Yoon, K., Pellaroni, L., Ramamoorthy, K., Gaido, K., and Safe, S. Ligand structure-dependent differences in activation of estrogen receptor α in human HepG2 liver and U2 osteogenic cancer cell lines. Mol. Cell. Endocrinol., *162:* 211–220, 2000.
40. Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., and Klinge, C. M. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β. Endocrinology, *141:* 3657–3667, 2000.
41. Freyberger, A., Hartmann, E., Hildebrand, H., and Krotlinger, F. Differential response of immature rat uterine tissue to ethinylestradiol and the red wine constituent resveratrol. Arch. Toxicol., *11:* 709–715, 2001.
42. Pommier, Y. Eukaryotic DNA topoisomerase I: genome gate keeper and its intruders, camptothecins. Semin. Oncol., *23:* 1–10, 1996.
43. Shao, R. G., Cao, C. X., Shimizu, T., O'Connor, P. M., Kohn, K. W., and Pommier, Y. Abrogation of an S-phase checkpoint and potentiation of camptothecin cytotoxicity by 7-hydroxystaurosporine (UCN-01) in human cancer cell lines, possibly influenced by p53 function. Cancer Res., *57:* 4029–4035, 1997.
44. Vannucchi, S., Percario, Z. A., Chiantore, M. V., Matarrese, P., Chelbi-Alix, M. K., Fagioli, M., Pelicci, P. G., Malorni, W., Fiorucci, G., Romeo, G., and Affabris, E. Interferon-β induces S phase slowing via up-regulated expression of PML in squamous carcinoma cells. Oncogene, *19:* 5041–5053, 2000.
45. Fredlund, J. O., and Oredsson, S. M. Normal G1/S transition and prolonged S phase within one cell cycle after seeding cells in the presence of an ornithine decarboxylase inhibitor. Cell Prolif., *29:* 457–465, 1996.
46. Schneider, Y., Vincent, F., Duranton, B., Badolo, L., Gosse, F., Bergmann, C., Seiler, N., and Raul, F. Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Lett., *158:* 85–91, 2000.
47. Fontecave, M., Lepoivre, M., Elleingand, E., Gerez, C., and Guittet, O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. FEBS Lett., *421:* 277–279, 1998.
48. Sun, N. J., Woo, S. H., Cassady, J. M., and Snapka, R. M. DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. J. Nat. Prod. (Lloydia), *61:* 362–366, 1998.
49. Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., and Zappia, V. Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem. Biophys. Res. Commun., *250:* 53–58, 1998.
50. Hsieh, T-C., Juan, G., Darzynkiewicz, Z., and Wu, J. M. Resveratrol increase 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.
51. 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.
52. Park, J. W., Choi, Y. J., Jang, M. A., Lee, Y. S., Jun, D. Y., Suh, S. I., Baek, W. K., Suh, M. H., Jin, I. N., and Kwon, T. K. Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. Cancer Lett., *163:* 43–49, 2001.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/61/16/6137/2487548/ch1601006137.pdf by guest on 10 November 2024