Induced growth of BG-1 ovarian cancer cells by 17β-estradiol or various endocrine disrupting chemicals was reversed by resveratrol via downregulation of cell cycle progression

NAM-HEE KANG1*, KYUNG-A HWANG1*, TAE-HEE KIM2, SANG-HWAN HYUN1, EUI-BAE JEUNG1 and KYUNG-CHUL CHOI1

1College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk;
2Department of Obstetrics and Gynecology, College of Medicine, Soonchunhyang University, Bucheon, Republic of Korea

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Abstract. Resveratrol (trans-3,4',5-trihydroxystilbene; RES), a phytoestrogen, exists in grape skin and red wine. Endocrine disrupting chemicals (EDCs) appear to promote the development and progression of estrogen-dependent cancers. In this study, we evaluated the inhibitory effect of RES on the cell proliferation induced by various EDCs in BG-1 ovarian cancer cells. Various EDCs, such as bisphenol A (BPA), nonylphenol (NP), octylphenol (OP), methoxychlor (MXC) and hexabromocyclododecane (HBCD), were employed in this study. In the in vitro experiments, treatment of BG-1 cells with E2, BPA, NP, OP, MXC or HBCD resulted in an increase of cell growth. By contrast, increased cell viability induced by these EDCs was reversed when co-cultured with RES. In addition, we examined the regulation of cell cycle-related genes by RT-PCR and western blot analysis. Treatment with each EDC was found to decrease only the gene expression of p21 and increase the expression of cell cycle-dependent kinase 2 (CDK2). However, co-treatment with RES and each EDC resulted in an increased gene expression of p21 and a decreased expression of CDK2. Cyclin D1 was increased by downregulating p21 only when treated with each EDC in the absence of RES, while co-treatment with RES and each EDC decreased the gene expression of cyclin D1 by upregulating p21. Taken together, RES appears to be an inhibitor of cyclin D1 and CDK2 and is responsible for the cell cycle arrest at the G1 phase. In addition, when co-treated with each EDC, RES increased the expression of p21 and resulted in the growth inhibition of BG-1 ovarian cancer cells. Taken together, these results indicate the antiproliferative effect of RES, a dietary phytoestrogen, on estrogen-dependent ovarian cancer cells activated by EDCs.

Introduction

Endocrine disrupting chemicals (EDCs) are the environmental chemicals that interfere with endocrine systems by adversely affecting hormone balance or disrupting normal function in the organs that hormones regulate or modulate (1). EDCs, such as bisphenol A (BPA), 4-nonylphenol (NP), 4-tert-octylphenol (OP), methoxychlor (MXC) and hexabromocyclododecane (HBCD), are released from industrial products, such as plastics, pesticides, detergents and other chemosynthetic products (2). EDCs lead to serious detrimental effects on the reproductive and developmental processes of human, animals and plants (3). They possess the potential to increase human health risks and affect the immune system and the development of vital organs. In addition, it has been reported that EDCs may increase the risk of cancer incidence (2). For example, the cell growth of MCF-7 breast cancer cells was increased by MXC and BPA in vitro (4). The cell proliferation of BG-1 ovarian adenocarcinoma cells was promoted by di-n-butyl phthalate (DBP) and HBCD through upregulation of the expression of cyclin-dependent kinase 4 (CDK4). These results suggest that DBP and HBCD have sufficient potency to disrupt the endocrine system and to stimulate cell growth in estrogen receptor (ER)-positive BG-1 cancer cells in vitro through the upregulation of genes that are associated with cell cycle progression (5). Consequently, these EDCs may be carcinogenic. Therefore, therapeutic tools for reducing the risk of carcinogenicity resulting from the exposure of EDCs are required.

Ovarian carcinoma is one of the most frequent gynecologic cancers, affecting more than 200,000 female individuals annually worldwide. Findings of a recent study showed that p21 effectively suppresses cancer growth by enhancing apoptosis and DNA damage response in ovarian cancer (7). Therefore, an investigation was conducted to identify an anticancer agent in natural food that enhances the expression of p21. Additionally, an anticancer therapy was examined for the cancers promoted...
by EDCs using resveratrol (RES) in the BG-1 ovarian adenocarcinoma cell line, a well-known estrogen-dependent cell line expressing ERs, including ERα and ERβ (3,6).

RES, a natural polyphenolic compound found in a variety of food sources, such as grapes, peanuts and red wine, is known for its anti-oxidant and anti-inflammatory effects, cardiovascular protection and increases longevity. RES has a strong chemopreventive effect (8) against the development of cancers, such as skin, breast, prostate, lung, pancreatic and ovarian cancer, by inhibiting cancer cell growth (9-13). RES also promotes the phosphorylation of p53 in a dose- and time-dependent manner in human breast cancer cells. Thus, as p53 is activated, it causes either cell cycle arrest or apoptosis. In addition, the expression of p21 is upregulated by the increased expression of p53, leading to cancer cell death. In this study, the inhibitory effect of RES on cell proliferation of the BG-1 ovarian adenocarcinoma cell line, which was stimulated by the exposure of EDCs, was also evaluated. EDCs, such as BPA, NP and OP, appeared to upregulate cyclin D1 and cyclin-dependent kinase 2 (CDK2), which is responsible for cell cycle progression. By contrast, RES increased the expression of p21, the CDK inhibitor, leading to the suppression of cancer cell growth.

Materials and methods

Reagents and chemicals. 17β-estradiol (E2), BPA, NP, OP, MXC, HBCD and RES were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Junsei Chemical Co., Tokyo, Japan) and stored as stock solutions at 4°C.

Cell culture. The BG-1 ovarian adenocarcinoma cell line was obtained from Dr K.S. Korach (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc.), 1% penicillin G, streptomycin (Cellgro Mediatech, Inc., Manassas, VA, USA) and 1% antifungal HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂-95% air. To prevent the effects of the estrogenic components of DMEM and FBS, phenol red-free DMEM supplemented with 5% charcoal-dextran-treated FBS (CD-FBS) was used to rule out the estrogenicity of EDCs in BG-1 cells. Cells were detached with 0.05% trypsin/0.02% EDTA in Mg²⁺/Ca²⁺-free Hank's balanced salt solution (HBSS; PAA Laboratories Ltd., Linz, Austria).

Cell proliferation assay. To examine the anticancer effect by RES, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. BG-1 cells were plated at 4,000 cells/well in 96-well plates in 100 µl of phenol red-free DMEM with 5% CD-FBS medium. After a 48-h incubation, the cells were washed and treated with RES at concentrations of 0, 10, 25, 50, 75 and 100 µM in phenol red-free DMEM with 0.1% of DMSO (Junsei Chemical Co.), respectively, for 5 days. Cell viability was assessed using an MTT assay. MTT (10 µl; Sigma-Aldrich Corp.) solution (5 mg/ml) was added to each well and the plates were incubated for 4 h at 37°C. Supernatants were removed and 100 µl of 99.0% DMSO (Junsei Chemical Co.) was added to each well to dissolve the resultant formazan crystals. Optical densities were measured at a wavelength of 540 nm using an ELISA-Reader (VERSA man; Molecular Devices, Sunnyvale, CA, USA).

To evaluate the cell proliferative effect on BG-1 cells, the various EDCs were treated. BG-1 cells were plated in 96-well plates of 4,000 cells/well in 100 µl of phenol red-free DMEM with 5% CD-FBS medium. After a 48-h incubation, the cells were washed and treated with DMSO, E2, BPA, NP, OP, HBCD, MXC and RES at concentrations of 0.1%, 10⁻⁶, 10⁻⁵, 10⁻⁴, 10⁻³ and 10⁻² M, respectively. Co-treatment of RES with each EDC was conducted at the time for 5 days and cell viability was assessed using MTT assay.

Total RNA extraction and cDNA synthesis. BG-1 cells were cultured at 3×10⁵ cells/well of 6-well plates and DMSO, E2, BPA, NP, RES and the combination of RES with each EDC were treated. Total RNA was extracted at various time-points (0, 6 and 24 h) using TRizol reagents (Invitrogen Life Technologies) according to the manufacturer's instructions. The concentration of total RNAs was measured by a spectrophotometer (Optizen, Mecasys, Dae-Jeon, Korea) at 260/280 nm. Total RNA (1 µg) was dissolved in diethyl pyrocarbonated-deionized water for cDNA synthesis (14).

Semi-quantitative reverse transcription (RT)-PCR. cDNA was synthesized from total RNAs by RT-PCR. Reaction mixture comprised murine leukemia virus reverse transcripase (M-MLV RT; iNtRON Biotechnology, Sungnam, Kyeonggi-do, Korea), 200 pM nonamer random primer, dNTPs, RNase inhibitor and RT buffer (all from iNtRON Biotechnology). cDNA synthesis was performed at 37°C for 1 h and at 95°C for 3 min. cDNA of p21, CDK2, cyclin D1 and GAPDH was amplified using each forward and reverse primer. Taq polymerase, PCR buffer, dNTP mixture and each cDNA template were conducted via PCR process, as described previously (5,14,15) (Table I). The RT-PCR products were run on a 1.5% agarose gel and gene bands were compared to 100-bp ladders (iNtRON Biotechnology). The gels were scanned and the density of the bands on the gel was quantified using Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (15,16).

Western blot analysis. Western blotting was performed to identify the protein expression of p21 and cyclin D1 in BG-1 cells. BG-1 cells were cultured at 1×10⁶ cells/100-mm dish and 0.1% DMSO, E2, BPA, RES and the combination of RES plus E2 or BPA were treated. Cells were suspended in 100 µl of 1X RIPA lysis buffer. The protein concentrations were determined using a bicinchoninic acid method. Total protein (50 µg) was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.). PVDF membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich Corp.) for 2 h at room temperature. The membranes were incubated with mouse monoclonal anti-p21 antibody (1:4,000; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-cyclin D1 antibody (1:2,000; Abcam, Hanam-city, Gyeonggi-do, Korea)
and mouse monoclonal anti-GAPDH antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. The membranes were subsequently probed with anti-mouse IgG-HRP conjugate secondary antibody (1:3,000; Santa Cruz Biotechnology). Target proteins were detected with a West-Q Chemiluminescent Substrate kit Plus (GenDEPOT, Barker, TX, USA).

Statistical analysis. Data were analyzed with GraphPad Prism software (San Diego, CA, USA). The in vitro data were shown as the means ± SD. Statistical analysis was performed using the one-way ANOVA followed by Dunnett’s multiple comparison test and T-test. P<0.05 was considered statistically significant (17,18).

Results

Effects of RES, E2, EDCs or their combinations on the cell proliferation of BG-1 cells. To evaluate the effect on cell proliferation by RES, BG-1 cells were cultured with a vehicle (DMSO 0.1%) or RES (10-100 µM) for 5 days. RES effectively reduced the cell viability of BG-1 cells in a dose-dependent manner (Fig. 1A). When treated with 100 µM of RES, the viability of BG-1 cells was decreased by 64% compared to that of the vehicle. The effects of E2, several EDCs and their combinations with RES were examined by MTT assay on the viability of BG-1 cells were cultured with a vehicle (10-100 µM) for 5 days. RES effectively reduced the cell viability of BG-1 cells in a dose-dependent manner (Fig. 1A). When treated with 100 µM of RES, the viability of BG-1 cells was decreased by 64% compared to that of the vehicle. The effects of E2, several EDCs and their combinations with RES were examined on the viability of cell viability of BG-1 cells by MTT assay. BG-1 cells were plated at 4,000 cells/well in 100 µl of phenol red-free DMEM with 5% CD-FBS medium. (A) The cells were washed and treated with RES at concentrations of 0, 10, 25, 50, 75 and 100 µM in phenol red-free DMEM with 0.1% of DMSO for 5 days. Values are the means ± SD. *Significant elevation compared to vehicle (0.1% DMSO), P<0.05 (Dunnett’s multiple comparison test). (B) Various EDCs were treated. After incubation for 48 h, the cells were washed and treated with DMSO, E2, BPA, NP, OP, HBCD, MXC and RES at concentrations of 0, 10, 25, 50, 75 and 100 µM in phenol red-free DMEM with 0.1% of DMSO for 5 days. Values are the means ± SD. *Significant elevation compared to vehicle (0.1% DMSO), P<0.05 (Dunnett’s multiple comparison test). #Significant elevation compared to E2 or each EDC treatment group, P<0.05 (t-tests). Co-treatment of RES with each EDC was conducted at the time for 5 days and cell viability was assessed by MTT assay. Values are the means ± SD. *Significant elevation compared to vehicle (0.1% DMSO), P<0.05 (Dunnett’s multiple comparison test). #Significant elevation compared to E2 or each EDC treatment group, P<0.05 (t-tests).
expression of p21 was significantly decreased by E2 and NP at 24 h, while its expression levels were significantly increased at 6 and 24 h in the presence of RES and its combinations (Fig. 3A). The cyclin D1 expression levels of E2 and NP were increased at 6 and 24 h, but markedly decreased by RES, E2 plus RES, or NP plus RES (Fig. 3B). The gene expression of CDK2 was considerably increased by E2 and NP at 6 and 24 h compared to the vehicle, but significantly decreased by RES and its combinations with E2 or NP (Fig. 3C).

Effects of E2, BPA, RES or their combinations on the protein expression of cell cycle-related genes. To investigate whether
E2, BPA, RES or their combinations alter the protein expression of cell cycle-related genes in the BG-1 human ovarian cancer cell line, western blot analysis was performed for the protein samples isolated from the cells at 0, 24 and 48 h in a time-dependent manner. The protein levels of p21 and cyclin D1 were measured following treatment with E2, BPA, RES, E2 plus RES or BPA plus RES in BG-1 cells. Findings showed the protein levels of p21 to be significantly decreased by E2 and BPA compared to the vehicle at 24 and 48 h, whereas the levels were markedly increased by RES, E2 plus RES, or BPA plus RES compared to the vehicle (Fig. 4). When treated with E2 plus RES, or BPA plus RES, the p21 protein exhibited a stronger expression compared to RES only at 24 h.

Finding of this study showed that BG-1 cell growth was promoted by E2 or various EDCs, including BPA, NP, OP, MXC and HBCD. These EDCs may induce BG-1 cell proliferation by affecting estrogen signaling via ERs. To evaluate the inhibitory effect of RES on the growth of BG-1 cells, RES was treated in a single treatment or in combination with E2 or other EDCs. Consequently, the cell viability and cell growth of BG-1 cells previously promoted by these EDCs were significantly suppressed by RES, which was considered to be associated with the regulation of cell cycle-related genes. Regulation of the cell cycle is closely involved in tumor cell proliferation, differentiation and apoptosis. Cell cycle progression is coordinately regulated by a number of protein factors that mainly include cyclins, CDKs and cyclin-dependent kinase inhibitors (CKIs). The regulation of CDK activation is crucial during cell cycle changes and is affected by cyclins (24). Cyclin D1 is an important protein for cell cycle progression and is known to function as a regulatory subunit of CDK4 or CDK6, whose activity is required for the G1/S-phase transition (23). The activities of CDKs are also inhibited by p21, p27 and p57, which have a nuclear localization signal at the C-terminus (24). This group of proteins exerts its inhibitory activities by interacting with a variety of cyclin-CDK complexes, thereby inducing S-phase arrest. Thus, the increased expression of p21 blocks the expression of the cell cycle progression factor and leads to increased G0/G1 cell cycle arrest. In addition, the increased p21 protein may contribute to enhanced cell apoptosis and genomic instability.

In this study, p21, cyclin D1 and CDK2 were observed as the main factors affecting cell viability or cell proliferation. The gene expression of cyclin D1 and CDK2 was considerably decreased by RES in a time-dependent manner. However, the gene expression of p21 was significantly increased by RES, which is considered to inhibit the expression of cell cycle progression factors, i.e., cyclin D1 and CDK2, and induce cell apoptosis through G0/G1 cell cycle arrest. The expression
patterns of p21 and cyclin D1 proteins by E2, EDCs and RES were detected concomitantly with their gene expression, i.e., the protein levels of p21 were significantly increased and cyclin D1 was markedly decreased by RES.

Our findings demonstrated that whereas E2 and several EDCs promoted the cell proliferation of ovarian adenocarcinoma BG-1 cells expressing ERs, RES effectively inhibited cell proliferation. As shown in this study, the cell proliferation of ovarian cancer cells by EDCs suggests that certain EDCs possess carcinogenic or cancer-promoting risks. However, RES showed antitumor activity on BG-1 ovarian cancer by reversing the cell proliferative effects induced by EDCs. Taken together, these results indicate that RES suppresses ovarian cancer cell growth by inducing cell cycle arrest. Although the cell cycle progression factors, i.e., cyclin D1 and CDK2, were inhibited, p21 protein, the cell cycle arrest factor that blocks a variety of cyclin-CDK complexes, was upregulated by RES. Therefore, we suggest the therapeutic effect and underlying mechanism of RES as a chemotherapeutic agent against estrogen responsive cancers whose cell growth may be induced by EDCs.

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References