Genistein, a soy phytoestrogen, prevents the growth of BG-1 ovarian cancer cells induced by 17β-estradiol or bisphenol A via the inhibition of cell cycle progression

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Abstract. An endocrine disrupting chemical (EDC) is a global health concern. In this study, we examined the effects of genistein (GEN) on bisphenol A (BPA) or 17β-estradiol (E2)-induced cell growth and gene alterations of BG-1 ovarian cancer cells expressing estrogen receptors (ERs). In an in vitro cell viability assay, E2 or BPA significantly increased the growth of BG-1 cells. This increased proliferative activity was reversed by treatment with ICI 182,780, a well-known ER antagonist, while cell proliferation was further promoted in the presence of propyl pyrazole triol (PPT), an ERα agonist. These results imply that cell proliferation increased by E2 or BPA was mediated by ERs, particularly ERα. BPA clearly acted as a xenoestrogen in BG-1 ovarian cancer cells by mimicking E2 action. In contrast, GEN effectively suppressed BG-1 cell proliferation promoted by E2 or BPA by inhibiting cell cycle progression. E2 and BPA increased the expression of cyclin D1, a factor responsible for the G1/S cell cycle transition. They also decreased the expression of p21, a potent cyclin-dependent kinase (CDK) inhibitor that arrests the cell cycle in G1 phase, and promoted the proliferation of BG-1 cells. As shown by its repressive effect on cell growth, GEN decreased the expression of cyclin D1 augmented by E2 or BPA. On the other hand, GEN increased the p21 expression downregulated by E2 or BPA. Collectively, our findings suggest that GEN, a dietary phytoestrogen, has an inhibitory effect on the growth of estrogen-dependent cancers promoted by E2 or BPA.

Introduction

Physiological estrogens are a group of steroid hormones that include estrone (E1), estradiol (E2), and estriol (E3). Although E3 is the most plentiful among these three factors, E2, also known as 17β-estradiol, exerts the strongest estrogenic effect. Estrogens are produced in ovaries, adrenal glands, and fat tissues, and function as the primary female sex hormones that promote the development of secondary sexual characteristics and regulate certain functions of the reproductive system. In addition, these compounds control various metabolic processes including bone growth, protein synthesis, and fat deposition. Estrogens have also been reported to be linked to the pathogenesis of several cancers in the reproductive organs. Previous studies have shown that circulating levels of estrogens may be most strongly associated with the risks of breast (1-4), ovarian (5-7), endometrial (8), and cervical (9) cancers. These diseases are known as estrogen-responsive or estrogen receptor (ER)-positive cancers because the actions of estrogen are mediated by ERs and ER expression has been observed in these cancers.

Recently, chemical compounds called endocrine disrupting chemicals (EDCs) are emerging as another risk factor for hormone-responsive cancers (10). EDCs are environmental substances that interfere with the biosynthesis, signaling, or metabolism of natural hormones in the body, thus having serious detrimental effects on reproductive and developmental processes (11). Xenoestrogens are classified as EDCs with estrogenic activity that disrupt normal estrogen signaling mediated by ERs (12-15). Bisphenol A (BPA) is a widely used industrial compound and a typical xenoestrogen (16,17). This chemical has been used for the manufacturing of polycarbonate plastics and polystyrene resins, and is commonly found in plastic bottles, plastic food containers, dental materials, and compounds used to coat containers for canned food. BPA can leach from these products in appreciable quantities, and thus humans are easily exposed to it through normal product use (18-20). After the estrogenic properties of BPA were discovered in 1930 (16), many studies published in the following decades have characterized the hazardous health effects of this compound and identified BPA as an endocrine disruptor. For instance, perinatal exposure to environmentally relevant concentrations of BPA causes morphological and functional alterations of the male and female genital tracts (21). In so doing, BPA may predispose the affected individuals to earlier onset of disease and reduced fertility, and induce neoplastic
transformation in human breast epithelial cells (21-23). Currently, the connection between perinatal BPA exposure and breast cancer is being examined (24).

In the present study, we examined the effect of BPA on the risk of ovarian cancer cell proliferation. Although this disease is one of the most frequently observed gynecologic cancers and is an estrogen-responsive disorder (25-27), the pathogenic actions of BPA on ovarian carcinoma have not been fully elucidated. Some previous reports suggest that BPA stimulates the proliferation of OVCAR-3 human ovarian cancer cells by inducing leptin receptor expression (28) or decreasing caspase-3 activity (29). To evaluate the effect of BPA on ovarian cancer development, we used the BG-1 ovarian adenocarcinoma cell line, an estrogen-dependent cell line that expresses ERs. In a cell proliferation assay, BPA promoted BG-1 cell growth as did E2, indicating that BPA acts as a xenoestrogen which has an obvious estrogenic effect on estrogen-responsive ovarian cancer. To explore ways to reverse the positive effects of BPA on cancer cell proliferation, we also examined the suppressive effect of genistein (GEN) on cell growth promoted by E2 or BPA. GEN is a classical phytoestrogen that is a plant-derived and naturally occurring dietary xenoestrogen which influences multiple biochemical functions (30). Based on epidemiologic observations indicating that incidences of cancer, including breast cancer, are much lower in Asian populations that consume significantly higher amounts of phytoestrogens compared to Western individuals, the chemoprotective properties of GEN have been extensively studied (31-33) although the anticancer effect of GEN remains unclear. Our present study showed that GEN effectively suppressed BG-1 ovarian cancer cell proliferation induced by E2 or BPA. These findings may be considered as an evidence of another chemopreventive activity of GEN that can nullify the carcinogenic risks associated with BPA, a potent chemoestrogenic EDC, or E2.

Materials and methods

Reagents and chemicals. 17β-estradiol (E2), BPA, and ICI 182,780 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). GEN was obtained from LC Laboratories (Woburn, MA, USA). Propyl pyrazole triol (PPT) and diarylpropionitrile (DPN) were purchased from Tocris (Ellisville, MO, USA). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Junsei Chemical Co., Tokyo, Japan) and stored as stock solutions at 4˚C.

Cell culturing. BG-1 ovarian adenocarcinoma cells were 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 and 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 estrogenic components in the DMEM and FBS, BG-1 cells were also cultured in phenol red-free DMEM supplemented with 5% charcoal-dextran treated FBS (CD-FBS) to measure the estrogenicity of the EDCs. Cells were detached with 0.05% trypsin/0.02% EDTA in Mg²⁺/Ca²⁺-free Hank's balanced salt solution (PAA Laboratories, Pasching, Austria).

Cell viability assay. To evaluate the effect of E2 or BPA on BG-1 cell proliferation, a cell viability assay was conducted as previously described (34-36). BG-1 cells were seeded at a density of 4,000 cells/100 µl of phenol red-free DMEM with 5% CD-FBS medium per well of 96-well plates. After incubating for 48 h, the cells were washed and treated with various concentrations of E2 or BPA (E2: 10⁻⁸⁻⁴ M, BPA: 10⁻⁴⁻² M) in phenol red-free DMEM supplemented with 0.1% DMSO for 5 days. DMSO was used as a vehicle and a negative control. Cell viability was assessed with the addition of 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution. MTT (10 µl of 5-mg/ml solution) was added to each well and the plates were incubated for 4 h at 37°C. Supernatants were removed and 100 µl of DMSO was added to each well to dissolve the resultant formazan crystals. The optical density (OD) of each well was measured at 540 nm using an ELISA reader (VERSA man, Molecular Devices, Sunnyvale, CA, USA) and used to calculate the number of viable cells as previously described (37,38). Viability of cells treated with the different EDCs was calculated relative to the control (DMSO-treated) cells.

To demonstrate the connection between E2 or BPA action and ER signaling, BG-1 cells were co-treated with E2 or BPA along with ICI 182,780 (a typical ER antagonist), PPT (an ERα agonist), or DPN (an ERβ agonist). The concentrations of ICI 182,780, PPT and DPN were 10⁻⁷, 10⁻⁸ and 10⁻⁹ M, respectively. To evaluate the effect of GEN on BG-1 cell proliferation, the cells were also co-treated with a combination of GEN and E2 or BPA. GEN was added at concentrations of 1.0, 2.5, 5.0, 7.5 and 10x10⁻⁵ M in the presence of 10⁻⁸ M of E2 or 10⁻⁵ M of BPA. After treating these reagents, identical experimental procedures were performed using MTT as in the treatment of E2 or BPA. All experiments were done at least three times.

Total RNA extraction. BG-1 cells were seeded at a density of 3.0x10⁴ cells per well in a 6-well plate, and then treated with DMSO, E2, BPA, or a combination of GEN and E2 or BPA. The concentrations of E2, BPA, and GEN were 10⁻⁸, 10⁻⁹ and 10⁻⁴ M, respectively. Total RNA was extracted at various time-points (0, 6, and 24 h) using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The concentration of total RNA was measured with a spectrophotometer (Optizem, Mecasys, Deajeon, Republic of Korea) at 260/280 nm. Total RNA (1 µg) was then dissolved in diethyl pyrocarbonated-deionized water (DEPC-DW) for cDNA synthesis.

Semi-quantitative reverse transcription (RT) PCR. cDNA was synthesized from total RNA by RT-PCR. The reaction mixture contained murine leukemia virus reverse transcriptase (M-MLV RT; iNtRON Biotechnology, Sungnam, Republic of Korea), 200 µM nonamer random primer (iNtRON Biotechnology), dNTPs (iNtRON Biotechnology), RNase inhibitor (iNtRON Biotechnology), and RT buffer (iNtRON Biotechnology). cDNA synthesis was performed at 37°C for 1 h and 95°C for 5 min. p21,
cyclin D1, and GAPDH cDNAs were amplified by PCR with specific forward and reverse primers, Taq polymerase, PCR buffer, and dNTP mixture, and each cDNA template as previously described. Sequences of the forward and reverse primers along with the predicted sizes of each gene product are shown in Table I. The RT-PCR products were separated on a 1.5% agarose gel and the size of each gene band was estimated by comparison with 100-bp size ladders (iNtRON Biotechnology). The gels were scanned and the band densities were quantified using Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were done at least three times.

Western blot analysis. Western blotting was performed to assess the protein expression of cyclin D1 and p21 in BG-1 cells. The cells were cultured to a density of 1.0ⅹ10^6 cells per 100-mm dish and then treated with DMSO, E2, BPA, or combinations of GEN and E2 or BPA for 24 and 48 h. The concentrations of E2, BPA, and GEN were 10^{-9}, 10^{-5} and 10^{-4} M, respectively. After treatment, the cells were suspended in 100 µl of 1X RIPA buffer (50 mM Tris-HCl, pH 8.0.; 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS). Total protein concentrations were determined using bicinchoninic acid (BCA; Sigma-Aldrich Corp.) and 50 µg of total protein were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.), and the membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich Corp.) for 2 h at room temperature. The membranes were then incubated with mouse monoclonal anti-p21 (1:4,000; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-cyclin D1 (1:2,000; Abcam, Hanam-city, Republic of Korea), or mouse monoclonal anti-GAPDH (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 2 h at room temperature. The membranes were subsequently probed with anti-mouse IgG HRP-conjugated secondary antibody (1:3,000; Santa Cruz Biotechnology) for 2 h at room temperature. Target proteins were detected with a West-Q Chemiluminescent Substrate Plus kit (GenDEPOT, Barker, TX, USA). All experiments were done at least three times.

Statistical analysis. All data were analyzed with GraphPad Prism software (San Diego, CA, USA). The in vitro data are presented as the mean ± SD. Statistical analyses were performed using a one-way ANOVA followed by Dunnett's multiple comparison test and Student's t-test. P-values <0.05 were considered to be statistically significant.

Results

Cell proliferation effect by E2 or BPA on BG-1 cells. To evaluate the effect of E2 or BPA on cell proliferation, BG-1 cells were cultured with vehicle (0.1% DMSO, control), E2 (10^{-9}-10^{-6} M), or BPA (10^{-8}-10^{-5} M) for 5 days. E2 effectively increased the viability of BG-1 cells at concentrations of 10^{-9}-10^{-7} M in a dose-dependent manner (Fig. 1A). At concentrations of 10^{-7} M...
and above, BPA also promoted cell proliferation (Fig. 1B). Although higher concentrations of BPA were needed to induce significant cell proliferation compared to E2, BPA was shown to exert an estrogenic effect on the BG-1 cells by mimicking E2 action.

**Effects of E2 or BPA on the proliferation of cells co-treated with ER modulators.** To determine whether increased cell proliferation promoted by E2 or BPA was mediated by ER signaling, BG-1 cells were co-treated with various ER modulators along with E2 or BPA and cell viability was measured. When the cells were co-treated with ICI 182,780 (a well-known ER antagonist) and E2 (10⁻⁹ and 10⁻⁸ M) or BPA (10⁻⁶ and 10⁻⁵ M), cell proliferation increased by treatment with E2 or BPA alone was dramatically reduced (Fig. 2). ICI 182,780, also called Fulvestrant, is an intact ER antagonist that does not exert any agonist effects, working both by downregulating and degrading the ER (39,40). Based on the result showing that E2 or BPA could not induce cell proliferation when the ER was inactivated by ICI 182,780, it was hypothesized that the proliferation of BG-1 cells was mediated by ER signaling via E2 or BPA.

We next determined which ER isoform, ERα or ERβ, was associated with the positive effect of E2 or BPA on cell proliferation. For this, the cells were co-treated with PPT or DPN (agonists of ERα and ERβ, respectively) and E2 or BPA. As shown in Fig. 3A and B, PPT in combination with E2 or BPA promoted BG-1 cell growth compared to a single treatment of E2 or BPA (for 10⁻¹¹ and 10⁻¹⁰ M of E2, and for 10⁻⁹, 10⁻⁸ and 10⁻⁷ M of BPA). On the other hand, DPN in combination with E2 or BPA had no effect on cell proliferation (Fig. 3C and D). These data showed that BG-1 cell proliferation was mainly mediated by ERα and thus E2 or BPA induced cell growth via ERα signaling.

![Figure 2](image2.png)

**Figure 2.** Viability of BG-1 human ovarian cancer cells following co-treatment with E2 or BPA and ICI 182,780, an ER antagonist. Cells were treated with DMSO (0.1 or 0.2%) as a control, E2 (10⁻⁹ and 10⁻⁸ M), or BPA (10⁻⁶ and 10⁻⁵ M) in the presence or absence of ICI 182,780 (10⁻⁷ M) for 5 days. Cell viability was measured with an MTT assay. Data represent the mean ± SD of triplicate experiments. *Significant difference between cells treated with E2 or BPA and the control (p<0.05 according to Dunnett’s multiple comparison test). #Significant reduction with co-treatment compared to treatment with E2 or BPA alone (p<0.05 according to Student's t-test).

![Figure 3](image3.png)

**Figure 3.** Viability of BG-1 human ovarian cancer cells following co-treatment with E2 or BPA and PPT, an ERα agonist, or DPN, an ERβ agonist. Cells were treated with DMSO (0.1 or 0.2%) as a control, E2 (10⁻¹¹, 10⁻¹⁰ and 10⁻⁹ M), or BPA (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M) in the presence or absence of PPT (10⁻⁸ M) or DPN (10⁻⁸ M) for 5 days. Cell viability was measured using an MTT assay. (A) The effect of E2 on cell proliferation in the presence or absence of PPT. (B) The effect of BPA on cell proliferation in the presence or absence of PPT. (C) The effect of E2 on cell proliferation in the presence or absence of DPN. (D) The effect of BPA on cell proliferation in the presence or absence of DPN. Data represent the mean ± SD of triplicate experiments. *Significant elevation in cell viability following treatments with E2, BPA, and a respective combination of PPT or DPN compared to the control (p<0.05 based on Dunnett’s multiple comparison test). #Significant elevation or reduction in cell viability by co-treatment compared to treatment with E2 or BPA alone (p<0.05 according to Student's t-test).
Anti-proliferative effect by GEN on E2 or BPA-induced cell proliferation. To evaluate the effect of GEN on cell proliferation promoted by E2 or BPA, BG-1 cancer cells were treated with a combination of E2 or BPA and GEN. GEN (5.0, 7.5, and 10x10^{-5} M with E2 or 2.5, 5.0, 7.5 and 10x10^{-5} M with BPA) strongly suppressed the cell growth induced by E2 (10^{-9} M) or BPA (10^{-5} M) as shown in Fig. 4. These findings demonstrate that GEN has an anti-proliferative effect and reduces cancer cell growth promoted by E2 or BPA.

Effects of E2 and BPA alone or in combination with GEN on mRNA expression of cell cycle-related genes. We next examined the mechanism underlying the effects of E2 and BPA (alone or in combination with GEN) on BG-1 cell proliferation through changes in the mRNA expression of cell cycle-related genes. For this, we performed semi-quantitative RT-PCR on total RNA samples isolated from the cells treated with E2 or BPA with or without GEN compared to the DMSO control (p<0.05 according to Dunnett’s multiple comparison test).
were significantly decreased by treatment with E2 or BPA for 6 h compared to the control. On the other hand, these mRNA levels were significantly increased by co-treatment with BPA or E2 and GEN for 6 and 24 h compared to exposure to E2 or BPA alone (Fig. 5B and D). Alterations in the expression of cell cycle-related genes such as cyclin D1 and p21 may explain the effect of E2 or BPA on cell proliferation and the anti-proliferative activity of GEN.

**Effects of E2 and BPA alone or in combination with GEN on the protein expression of cell cycle-related genes.** To confirm that E2 and BPA altered the expression of genes involved in cell cycle regulation, we conducted a western blot analysis using antibodies specific for cyclin D1 and p21. As shown in Fig. 6, the protein levels of cyclin D1 were increased by E2 or BPA after 24 h of treatment compared to the control. These levels were decreased by co-treatment with GEN for 24 and 48 h compared to treatment with E2 or BPA alone (Fig. 6A and B). On the other hand, the protein expression of p21 was reduced by E2 or BPA after 24 h compared to the control. This effect was reversed by a co-treatment with GEN compared to treatment with E2 or BPA alone (Fig. 6A and C). These findings coincided with the changes we observed in mRNA expression and further validate the effect of E2 or BPA on cell proliferation and the anti-proliferative activity of GEN.

**Discussion**

It was recently found that estrogens are important factors in the initiation and progression of cancers, including breast and ovarian carcinomas. Since then, there has been a growing...
concern that EDCs, especially xenoestrogens, might potentially have carcinogenic effects on estrogen-sensitive organs (1,2,5-7,41). In the present study, we demonstrated that both E2 and BPA, a typical xenoestrogen, have the capacity to stimulate ovarian cancer cell proliferation. When added to cell culture medium devoid of estrogenic compounds, E2 and BPA significantly promoted the proliferation of BG-1 ovarian cancer cells. This increased cell proliferation was reversed by co-treatment with ICI 182,780, a well-known ER antagonist (39). Therefore, it was determined that E2 mediated the growth of BG-1 cells via ER signaling, and BPA exerted an estrogenic effect by mimicking E2 action.

Estrogen signaling is mainly mediated via two subtypes of ERs, ERα and ERβ, that are differentially expressed in various tissues and have unique functions (22,42). There is a careful balance between the actions of these two distinct receptor isoforms (43). Both have been reported to affect cellular proliferation and cell cycle events (44). However, ERβ may have an inhibitory effect on G2 and M phases of the cell cycle (43) whereas ERα was shown to be linked to cell cycle progression through the stimulation of cyclin D1 gene expression and induction of cell proliferation (44). cyclin D1 is a key regulator of the cell cycle that acts by binding to the retinoblastoma (Rb) protein and directing CDK4 and CDK6 to hyperphosphorylate Rb, leading to the progression from the G1 to S phase and cell growth (45). It was reported that E2-ERα mediates the dissociation of p21, a CKD inhibitor, from the cyclin E-CDK2 complex, the activation of cyclin-CDK complexes, and passage from the G1 to S phase (46). E2 was also found to enhance ERα binding to p53, a major tumor suppressor, and inhibit p21 transcription (47). Based on these findings, it can be said that E2 manipulates cell cycle progression and the proliferation rate of cancer cells by modulating the activities of cyclin-CDK complexes during G1 phase (43,46). In agreement with this hypothesis, E2 and BPA were shown in the present study to induce BG-1 cell proliferation by upregulating cyclin D1 and downregulating p21 via ERα signaling (Fig. 7). Interaction between ERα and E2 or BPA was implied based on the finding that increases in cell proliferation by E2 or BPA were further augmented by a co-treatment with PPT, an ERα agonist (48), but not by DPN, an ERβ agonist (48). These data showed that BPA acts as a distinct xenoestrogen in BG-1 ovarian cancer cells by mimicking E2 through similar mechanisms. In our previous study (49), we also examined the estrogenic effect of BPA mediated by gene expression alterations in BG-1 ovarian cancer cells using a microarray analysis. We found that BPA induces the transcription of E2-responsive genes such as RAB31, cyclin D1, cdk-4, insulin-like growth factor binding protein 4, and anti-mullerian hormone in a manner similar to E2.

In the present study, we also demonstrated the anticancer activity of GEN, a typical phytoestrogen, against carcinogenicity resulting from treatment with E2 or BPA. GEN is the most abundant isoflavone in soybean products and is known to have various biological activities (33). Among these, its anticancer effects against a diverse number of cancers including breast and prostate carcinomas have been considered to be most noteworthy (32,33). In the present study, we performed a cell viability assay to evaluate the effects of co-treatment with GEN and E2 or BPA. GEN effectively suppressed BG-1 ovarian cancer cell proliferation promoted by E2 or BPA. This anti-proliferative effect of GEN was achieved by reversing the effects of E2 or BPA on the expression of cell cycle-related genes. Unlike the actions of E2 or BPA, GEN suppressed the expression of cyclin D1 and enhanced the expression of p21 when administered with E2 or BPA, thereby leading to cell cycle arrest in G1 phase (Fig. 7). Further studies are required to understand the mechanisms underlying the anti-proliferative activities of GEN. In particular, elucidating the impact of GEN on ER signaling in estrogen-responsive cancers will be helpful for explaining the neutralizing or inhibitory effect of GEN on cancer progression induced by diverse types of EDCs.

In conclusion, our findings demonstrated that GEN, although classified as a natural xenoestrogen, acts as a chemopreventive agent by abolishing the carcinogenic risks associated with BPA, a potent chemosynthetic EDC, and E2.

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