Grape Seed Extract Is an Aromatase Inhibitor and a Suppressor of Aromatase Expression

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Abstract

Aromatase is the enzyme that converts androgen to estrogen. It is expressed at higher levels in breast cancer tissues than normal breast tissues. Grape seed extract (GSE) contains high levels of procyanidin dimers that have been shown in our laboratory to be potent inhibitors of aromatase. In this study, GSE was found to inhibit aromatase activity in a dose-dependent manner and reduce androgen-dependent tumor growth in an aromatase-transfected MCF-7 (MCF-7aro) breast cancer xenograft model, agreeing with our previous findings. We have also examined the effect of GSE on aromatase expression. Reverse transcription-PCR experiments showed that treatment with 60 μg/mL of GSE suppressed the levels of exon I.3–, exon PII–, and exon I.6–containing aromatase mRNAs in MCF-7 and SK-BR-3 cells. The levels of exon I.1–containing mRNA, however, did not change with GSE treatment. Transient transfection experiments with luciferase-aromatase promoter I.3/II or I.4 reporter vectors showed the suppression of the promoter activity in a dose-dependent manner. The GSE treatment also led to the down-regulation of two transcription factors, cyclic AMP-responsive element binding protein-1 (CREB-1) and glucocorticoid receptor (GR). CREB-1 and GR are known to up-regulate aromatase gene expression through promoters I.3/II and I.4, respectively. We believe that these results are exciting in that they show GSE to be potentially useful in the prevention/treatment of hormone-dependent breast cancer through the inhibition of aromatase activity as well as its expression. (Cancer Res 2006; 66(11): 5960-7)

Introduction

Aromatase catalyzes three consecutive hydroxylation reactions converting C-19 androgen to C-18 estrogen, which is the last and critical step of estrogen synthesis. The female hormone, estrogen, is known to stimulate breast cancer cell growth by binding to the estrogen receptor (ER). Therefore, aromatase and ER are being targeted as a treatment for breast cancer. Aromatase is expressed in a number of tissues such as ovary, placenta, adipose, and bone. The expression of aromatase in breast cancer tissue has been shown by enzyme activity measurement (1–3), immunocytochemistry (4–6), and reverse transcription-PCR (RT-PCR) analysis (7–9). Interestingly, aromatase is found to be expressed at higher levels in breast cancer tissue than normal breast tissues (7–9). The *in situ* produced estrogen, due to overexpressed aromatase in breast cancer cells, is thought to play a more crucial role in stimulating cancer cell growth than circulating estrogen (2).

The human aromatase gene contains nine translated exons (II-X) and at least eight untranslated exon I's (I.1, I.2, I.3, I.4, I.5, I.6, I.7, and PII). The translation start site is positioned in exon II and one of the 5′-untranslated exon I's of aromatase mRNA is spliced onto a common splicing junction of exon II, upstream of the translation start site (10–12). It has been found that the various untranslated exon I's are present at different levels in different aromatase-expressing tissues and cells (13–15). Studies conducted in this and other laboratories have revealed that exons I.3 and PII are the major exon I's in aromatase mRNA isolated from breast cancer cells, indicating that promoters I.3 and II are the major promoters driving aromatase expression in breast cancer (7, 14, 16, 17). Promoter I.4 is the major promoter expressed in breast stromal cells (14, 16).

Promoter II is a gonad-specific ovarian promoter regulated by many factors such as follicle stimulating hormone, cyclic AMP (cAMP), and SF-1 (18). Promoters I.3 and II are ~200 bp apart from each other and are the most proximal promoters to the translation start site. Promoter I.3 can also be stimulated by cAMP, as in the case of promoter II, and regulated by many factors including transforming growth factor-β (19, 20). Previous research from our laboratory identified two regulatory regions located near these two promoters. The first region, S1, is located between the two promoters. The trans-factors, which interact with this element, were found to be mostly nuclear receptors (21, 22). The second region found was a cAMP-responsive element (CREaro), located upstream of promoter I.3 (23). CREaro acts as an enhancer element when CREB-1 binds (24). CREB-1 expression is reported to be approximately five times higher in tumor-bearing adipose tissue than in normal breast adipose tissue (25).

Promoter I.4 is located ~70 kb upstream of exon II. It is a TATA-less promoter regulated by glucocorticoid, IFN-γ, and a number of class I cytokine family members such as interleukin (IL)-6 and IL-11 (20, 26, 27).

Our group previously reported that procyanidin B dimers purified from red wine were potent competitive inhibitors of aromatase. Furthermore, procyanidin B dimers were found to reduce androgen-dependent tumor growth in an aromatase-transfected MCF-7 (i.e., MCF-7aro) breast cancer xenograft model (28). This suggests that procyanidin B dimers suppress *in situ* estrogen formation. Procyanidin B dimers are rich in commercially available grape seed extract (GSE). GSE is composed of ~74% to 78% proanthocyanidins and <6% free flavanol monomers such as catechin, epicatechin, and their gallic acid esters. Proanthocyanidins in GSE are known to possess powerful protective properties against free radicals and oxidative stress and have been reported to exhibit a wide range of biological advantages such as antibacterial, antiviral, anti-inflammatory, and antiallergic actions. GSE has also been linked to cancer prevention/therapy because it down-regulated growth factor receptor signaling in prostate

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cancer DU145 cells (29) and exhibits cytotoxicity to MCF-7 breast cancer, A-427 lung cancer, CRL-1739 gastric adenocarcinoma, and K562 leukemia cells, but not to normal cells (30).

In this study, results have been generated to indicate that GSE suppresses not only aromatase activity but also aromatase promoter activity and expression. Whereas the inhibition of aromatase activity by GSE is expected, the finding on the suppression of aromatase expression by GSE is considered to be novel. Our experiments have further revealed that the GSE treatment also led to down-regulation of two transcription factors, CREB-1 and glucocorticoid receptor (GR), which are known to up-regulate aromatase gene expression through promoters I.3/II and I.4, respectively.

Materials and Methods

Cell culture. SK-BR-3 and MCF-7 breast cancer epithelial cells were purchased from American Type Culture Collection (Manassas, VA). SK-BR-3 cells were maintained in McCoy's 5A medium containing 2 mmol/L L-glutamine, 1× penicillin-streptomycin, 1× nonessential amino acid, 1 mmol/L sodium pyruvate, and 15% fetal bovine serum (FBS). MCF-7 cells were maintained in Eagle's MEM containing 2 mmol/L L-glutamine, 1× penicillin-streptomycin, 1× nonessential amino acid, 1 mmol/L sodium pyruvate, and 10% FBS. MCF-7aro cells are aromatase-overexpressing MCF-7 cells (31, 32) and are maintained in the same medium used for MCF-7 cells plus 100 μg/mL of G418. All the cell lines were cultured at 37°C with 5% CO_2.

"In-cell" aromatase assay. Aromatase activity was measured in a $[^3H]H_2O$ release assay as previously described (31). Tritiated [1β-^{3}H(N)] androstenedione (final concentration, 100 nmol/L) and 0.5 μL of 1 mmol/L progesterone per milliliter of medium were mixed with FBS-free culture medium. The substrate, androst-4-ene-3,17-dione [1β-^{3}H(N)] (specific activity 24.7 Ci/mmol), was purchased from New England Nuclear (Boston, MA). GSE at 20, 40, or 60 μg/mL was also added to the cells and incubated for 1 hour at 37°C and 5% CO_2. GSE was prepared by dissolving ActiVin GSE powder (San Joaquin Valley Concentrates, Fresno, CA) into water at varying desired concentrations. Following incubation, 1-mL medium samples were transferred into tubes containing a charcoal-dextran pellet, mixed by vortexing for 30 seconds, and then centrifuged at 15,000 × *g* for 10 minutes. For each sample, a 300-μL aliquot of supernatant was mixed with 3 mL of Scintisafe 30% liquid scintillation cocktail (Fisher, Pittsburgh, PA) and counted in an LS 6500 Multipurpose Scintillation Counter (Beckman Coulter, Fullerton, CA). One milliliter of 0.5 N NaOH was added to each well and the plates were shaken overnight at room temperature to solubilize cell proteins. Protein concentrations were determined to normalize measured radioactivity (counts per minute).

Animal experiment. Five- to six-week-old female BALB/c *nu/nu*, athymic, ovariectomized mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). At 8 weeks of age, the mice were s.c. implanted with a 7.5 mg/60-day time-release androstenedione pellet (Innovative Research of America, Sarasota, FL). Starting at 9 weeks of age, the mice were gavaged daily for 42 consecutive days with 100 μL of water containing 500 or 750 μg of GSE. Control mice were gavaged with 100 μL of water only. At 10 weeks of age, mice were given two s.c. injections of MCF-7aro cells (in 0.2 mL). The MCF-7aro cells were harvested from monolayer culture and resuspended in an equal volume of Matrigel (BD Biosciences, San Jose, CA) to a final concentration of 1×10^7/0.2 mL. Body weights were monitored weekly as an indicator of the animals' overall health. After 42 days of gavage treatment, the mice were euthanized and the tumors were removed and weighed.

RNA isolation and semiquantitative RT-PCR analysis of aromatase exon I usage. MCF-7 and SK-BR-3 cells were treated in triplicate with water, or 30 or 60 μg/mL of GSE for 24 hours in phenol red–free medium containing 10% charcoal-dextran–treated FBS. Total RNA was isolated by using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. The quality of RNA was assessed by electrophoresis on an RNA denaturing agarose gel. Reverse transcription was done at a final volume of 40 μL. Ten micrograms of total RNA were denatured at 70°C for 10 minutes with 50 ng of random primer (Invitrogen) and reverse transcribed in a mixture consisting of 0.5 mmol/L deoxynucleotide triphosphate (dNTP) mix, 40 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL), 80 units of RNase inhibitor (RNasin, Promega, Madison, WI), and reaction buffer supplied with the reverse transcriptase [25 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L DTT, and 5 mmol/L $MgCl_2$] for 45 minutes at 42°C, followed by heat inactivation for 15 minutes at 70°C. Then, 1 μL of cDNA was subjected to PCR amplification in a 25-μL reaction mixture containing 0.25 units of ChromaTaq DNA polymerase (Denville Scientific, Inc., Metuchen, NJ), 1× ChromaTaq reaction buffer, 0.2 μmol/L of each primer, 2 mmol/L of $MgCl_2$, and 0.2 mmol/L of dNTP mix. Exon I–specific RT-PCR analyses were done to examine the usage of exon I in the human aromatase gene as previously described (16, 33). Briefly, a reverse primer derived from exon II was used with forward primers in exons I.1, I.3, I.4, I.5, I.6, PII, and II for the amplification of exons I.1, I.3, I.4, I.5, I.6, PII, and II, respectively. PCR was conducted for 35 cycles (exons I.1, I.3, I.4, I.5, and II) or 30 cycles (exons I.6 and PII) using the following thermal profile: 94°C, 1 minute (denaturing); 54°C, 1 minute (annealing); and 72°C, 1 minute (extension). An additional 7-minute extension cycle was carried out before cooling the reaction mixture to 4°C. As an internal control to normalize aromatase mRNA expression in each sample, a set of human β-actin specific primers was used. Each PCR product was electrophoresed on a 1.2% agarose gel and stained with ethidium bromide.

Plasmid preparation. The genomic DNA was prepared from MCF-7 cells by using lysis buffer [100 mmol/L Tris-HCl (pH 8.5), 5 mmol/L EDTA, 0.2% SDS, 200 mmol/L NaCl, 100 μg proteinase K] and isopropanol. Aromatase gene promoter I.3/II and promoter I.4 constructs were prepared by PCR of MCF-7 genomic DNA. The genomic DNA fragments, −329/+284 bp of human aromatase promoter I.3/II (exon I.3 start site as +1) and −1,020/+350 bp of promoter I.4 (exon I.4 start site as +1), were subcloned into *Kpn*I/*Xho*I sites of the pGL3-Basic reporter vector (Promega). Both of the plasmids were confirmed for their accuracy through sequencing.

Transient transfection and reporter gene assay. MCF-7 and SK-BR-3 cells were transfected with pGL3basic-pI.3/II or pGL3basic-pI.4 using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. Cells (1.5×10^5 per well) were seeded onto 24-well plates and incubated overnight. A mixture of 1 μg of the reporter plasmid, 1.5 μL of Lipofectamine 2000, and OptiMEM (Invitrogen) was added into each well after the removal of the overnight-cultured medium and incubated for 6 hours at 37°C with 5% CO_2. After the 6-hour incubation, OptiMEM containing Lipofectamine 2000 and DNA was removed and the cells were cultured with various amounts of GSE in phenol red–free medium with 10% charcoal-dextran–treated FBS. After a 24-hour incubation following the transfection, the cells were lysed in 1× Reporter Lysis Buffer (Promega). After the centrifugation of the lysed cells, aliquots were used for luciferase activity assay and protein assay. Luciferase activity was measured using the Luciferase Assay System (Promega) and read in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Protein concentration was measured by the method of Bradford (ref. 34; Bio-Rad, Hercules, CA). The relative luciferase activity was calculated by dividing the light unit of luciferase activity by protein concentration of each sample.

Real-time quantitative PCR. Quantitative analyses of CREB-1 and GR mRNA expression levels were assessed using real-time PCR technique. One microliter of each cDNA sample prepared from GSE-treated MCF-7 and SK-BR-3 cells as indicated above were mixed in a total of 25-μL reaction volume with 1× iQ SYBR Green Supermix (Bio-Rad) and 0.2 μmol/L of each primer [CREB-1, 5′-AGCCACTCAGCCGGGTACTA-3′ (forward) and 5′-TAGTGGGTGCTGTGCGAATC-3′ (reverse), or GR, 5′-TGCCAAGGATCTGGAGATGA-3′ (forward) and 5′-GAGGAGCTGGATGGAGGAGA-3′ (reverse)]. The PCR thermal profile consisted of an initial incubation of 3 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. After the completion of the 40 cycles, two additional incubations of 1 minute at 95°C and 1 minute at 55°C were done followed by 81 cycles of 30 seconds of increasing temperatures starting at 50°C (melt curve). Amplification,

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detection, and data analysis were done with an iCycler IQ5 real-time detection system (Bio-Rad). Each sample was analyzed in triplicate. The relative expression levels of each gene were calculated by dividing the expression level of CREB-1 or GR by the expression level of β-actin for each sample.

Western blotting. MCF-7 and SK-BR-3 cells were cultured in 100-mm culture dishes and incubated with water or 60 μg/mL of GSE in phenol red–free medium containing 10% charcoal-dextran–treated FBS for 24 hours. The cells were lysed in SDS lysis buffer [75 mmol/L Tris-HCl (pH 6.8), 3% SDS, 15% glycerol] and whole-cell lysates were boiled for 5 minutes, cooled, and then sonicated briefly to reduce viscosity. Protein concentration was determined and samples were stored at −70°C until use. Twenty-five micrograms of proteins for each sample were boiled with 1× loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 minutes. Samples were then electrophoresed in 10% SDS-polyacrylamide gel and transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 100 mA for 90 minutes. The membrane was blocked for 2 hours with 5% nonfat milk in 1× TBS-T at room temperature to reduce background and then incubated with primary antibody, anti-CREB-1 or anti-GR (Santa Cruz Biotechnology, Santa Cruz, CA), in 5% bovine serum albumin at 4°C overnight. Anti-CREB-1 and anti-GR were used at 1:1,000 and 1:200 dilutions, respectively. After the membrane was incubated with the primary antibody and washed four times with 1× TBS-T for 5 minutes each wash, it was incubated with the secondary antibody, ImmunoPure Goat Anti-Rabbit IgG, Peroxidase Conjugated (Pierce, Rockford, IL) at 1:20,000 dilution in 5% nonfat milk for 60 minutes at room temperature. The membrane was washed four times again for 5 minutes each time with 1× TBS-T and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the protocol of the manufacturer. The membrane blot was exposed to Basic Autorad Film (ISC Bioexpress, Kaysville, UT) and developed using a Konica SRX-101A (Konica, Tokyo, Japan). The blot was reprobed with antiactin (Santa Cruz Biotechnology) at 1:1,000 dilution as a loading control.

Results

Suppression of aromatase enzymatic activity and tumor growth in mice xenograft model by GSE. Our group previously reported that procyanidin B dimers purified from red wine are potent inhibitors of aromatase. Furthermore, procyanidin B dimers were shown to reduce androgen-dependent tumor growth in an aromatase-transfected MCF-7 (i.e., MCF-7aro) breast cancer xenograft model but these chemicals did not affect the estrogen-dependent growth of MCF-7 tumors (28). These results indicate that procyanidin B dimers suppress *in situ* estrogen formation. Procyanidin B dimers are known to be present at high levels in grape seeds. To evaluate whether GSE is also effective at inhibiting aromatase, in-cell aromatase assay was conducted. The anti-aromatase effect of GSE was examined using MCF-7aro cells. GSE at 40 and 60 μg/mL inhibited aromatase activity by 70.4% and 80.5%, respectively (Fig. 1). Next, we examined whether GSE has the ability to suppress aromatase-expressing tumor growth in a mouse xenograft model using BALB/c *nu/nu*, athymic, ovariectomized mice carrying MCF-7aro tumor. Mice were given two s.c. injections of MCF-7aro cells (1×10^7 per site) that proliferate in an androgen-dependent manner. Mice were individually gavaged with the water control, or 500 or 750 μg GSE dissolved in 100 μL water for 42 consecutive days. At the end of the 42-day gavage treatment, mice were euthanized and tumors were removed and weighed. It was found that mice gavaged with 750 μg GSE had a significant reduction in tumor growth when compared with the control mice gavaged with water ($P = 0.0008$; Fig. 2). The average tumor weights for each group were 92.6 ± 39.9 mg (control), 77.7 ± 77.6 mg (500 μg GSE), and 47.9 ± 21.4 mg (750 μg GSE). These results showed that GSE intake could suppress the aromatase-positive tumor growth in mice, agreeing with our previous results from studies using procyanidin dimer preparations from red wine (28).

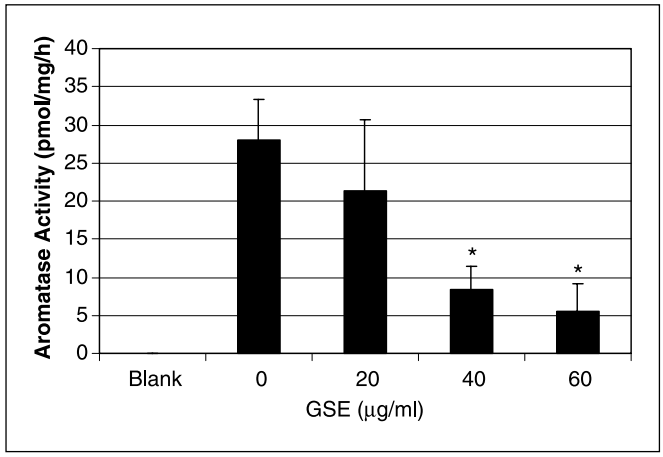

Figure 1. Inhibitory effect of GSE on aromatase activity. MCF-7aro cells were incubated with tritiated [1β-^{3}H(N)] androstenedione and increasing amount of GSE. Aromatase activity was measured by [^{3}H]H_2O release assay. Differences compared with non-GSE-treated control where $P < 0.01$ were considered statistically significant ($P = 0.0051$ for 40 μg/mL and $P = 0.0037$ for 60 μg/mL). Statistical analyses were done using the two-tailed Student's *t* test. Blank shows background activity where aromatase activity was measured from the wells without cells.

Suppression of aromatase expression by GSE. After we found GSE to be an aromatase inhibitor, a question was raised about whether GSE could also modulate the expression of aromatase. To answer this question, RT-PCR analysis of total aromatase mRNA (using primers from the exon II region) in GSE-treated breast cancer cells and vehicle-treated cells was done. When we learned that GSE could suppress aromatase expression at the transcriptional level, we further determined which exon I's/promoters are affected by GSE treatment by exon I–specific RT-PCR analyses in which a reverse primer derived from exon II was used with forward primers from exon I.1, I.3, I.4, I.5, I.6, or PII for the amplification of exon I.1, I.3, I.4, I.5, I.6, or PII, respectively. Human breast cancer epithelial cell lines MCF-7 and SK-BR-3 and human hepatoblastoma cell line HepG2 were chosen for this experiment. Aromatase mRNA in MCF-7 cells mainly contain exons I.3, I.6, and PII; SK-BR-3 cells mainly contain exon I.1, I.3, I.6, and PII; and HepG2 cells mainly contain exon I.4 (16, 33). Each set of three dishes was prepared and treated with 15 or 60 μg/mL of GSE or with water as the negative control. However, HepG2 cells were highly sensitive to GSE and we failed to obtain enough amount of total RNA for RT-PCR experiments. The level of exon II–containing mRNA was decreased on GSE treatment in both MCF-7 and SK-BR-3 cells (Fig. 3). It was found that total aromatase mRNA levels were decreased by GSE treatment. GSE at 60 μg/mL suppressed the levels of exon I.3–, PII–, and I.6–containing mRNAs in MCF-7 and SK-BR-3 cells. The levels of exon I.1–containing mRNA, however, did not change on GSE treatment. Exons I.4 and I.5 were not expressed in MCF-7 or SK-BR-3 cells (data not shown). The results showed that GSE could suppress aromatase gene transcription through down-regulation of promoters I.3/PII and I.6 in MCF-7 and SK-BR-3 cells.

Suppression of aromatase promoter activity by GSE. To examine whether GSE modulates human aromatase promoter activity, transient transfection experiments using aromatase

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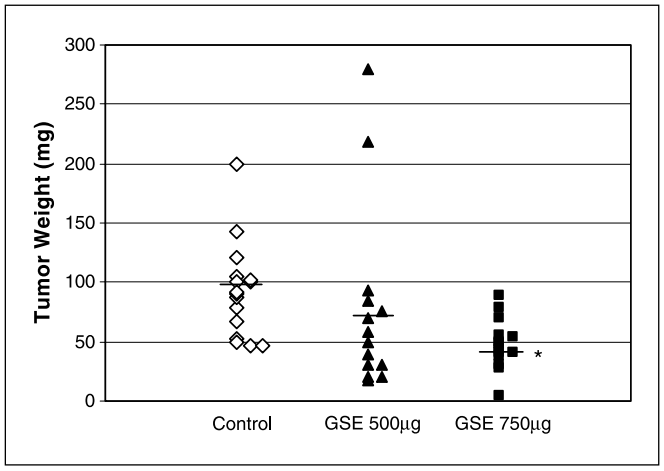

Figure 2. Suppression of MCF-7aro tumor growth in nude mice by GSE. Eight-week-old female BALB/c *nu/nu*, athymic, ovariectomized mice were s.c. implanted with a 7.5 mg/60-day time-release androstenedione pellet. Starting at 9 weeks of age, the mice were gavaged daily for 42 consecutive days with 100 μL water control, or 500 μg or 750 μg of GSE in 100 μL of water. At 10 weeks of age, mice were given two s.c. injections of 1×10^7 MCF-7aro cells per site. At the end of 42 days of daily gavage treatment, the mice were euthanized and the tumors were removed and weighed. *, $P = 0.0008$, compared with water control group (two-tailed Student's t test).

promoter reporter vectors were done. Because aromatase mRNAs in the breast stromal cells are exon I.4-dominant whereas exons I.3/II are the two major exon I's present in aromatase mRNAs isolated from breast tumors, we have been interested in the regulatory mechanisms of these three promoters. Therefore, promoter I.4 was also evaluated besides promoters I.3/II for this experiment. MCF-7 and SK-BR-3 cells were transfected with 1 μg of pGL3basic-pI.3/II or pGL3basic-pI.4 reporter vectors. pGL3basic-pI.3/II vector contains −329/+284 bp region (+1 as exon I.3 start site) of aromatase promoter I.3/II and pGL3basic-pI.4 vector contains −1,020/+350 bp region (+1 as exon I.4 start site) of aromatase promoter I.4. After 24-hour incubation with water (control), or 3.75, 7.5, 15, 30, or 60 μg/mL of GSE, the cells were lysed and subjected to assays for luciferase activity and protein concentration. Aromatase promoter I.3/II activity was suppressed by GSE in both MCF-7 and SK-BR-3 cells in a dose-dependent manner (Fig. 4*A*). GSE at 60 μg/mL decreased promoter I.3/II activity to 23.7% and 5.4% in MCF-7 and SK-BR-3 cells, respectively. Concentrations of 15, 30, and 60 μg/mL of GSE showed significant reduction in promoter I.3/II activity in MCF-7 cells ($P \lesssim 0.01$) whereas all of the concentrations tested showed significant reduction in SK-BR-3 cells ($P < 0.05$ for 3.75 μg/mL, $P < 0.01$ for others). GSE also suppressed promoter I.4 activity in both MCF-7 and SK-BR-3 cells (Fig. 4*B*). GSE at 60 μg/mL decreased promoter I.4 activity to 34.5% and 17% in MCF-7 and SK-BR-3 cells, respectively. Significant reduction was seen at the concentrations of 30 and 60 μg/mL in MCF-7 cells ($P < 0.05$ and $P < 0.01$ respectively) and at 15 through 60 μg/mL in SK-BR-3 cells ($P < 0.01$). These results indicated that GSE exhibits an inhibitory effect towards aromatase promoter I.3/II and I.4 activity and that GSE seems to show a stronger suppressive effect on aromatase promoter activity in SK-BR-3 than in MCF-7 cells. Procyanidin B1 and B2 dimers and the monomers, such as catechin, epicatechin, and epicatechin gallate, were examined and found not to be active in the inhibition of the activity of promoter I.3/II or I.4 (data not shown).

Down-regulation of GR and CREB-1 levels on GSE treatment. Promoter I.3/II is known to be up-regulated by cAMP through binding of CREB-1 to CRE within the promoters. On the other hand, promoter I.4 is known to be up-regulated by glucocorticoid but not by cAMP. To understand the mechanism of the down-regulation of aromatase expression and promoter I.3/II and I.4 activity, we decided to determine whether GSE treatment of MCF-7 and SK-BR-3 cells decreased the expression level of CREB-1 and GR. Real-time quantitative PCR and Western blotting experiments were done to determine mRNA and protein levels of the two transcription factors. GSE at 60 μg/mL significantly decreased CREB-1 mRNA expression level to 17.1% compared with water-treated control in MCF-7 cells (Fig. 5*A*). GSE concentrations of 15 and 60 μg/mL were tested on SK-BR-3 cells and showed significant suppressive effect in a dose-dependent manner (Fig. 5*A*). GSE at 60 μg/mL reduced CREB-1 mRNA expression to 22.7% in SK-BR-3 cells. GR mRNA level is also

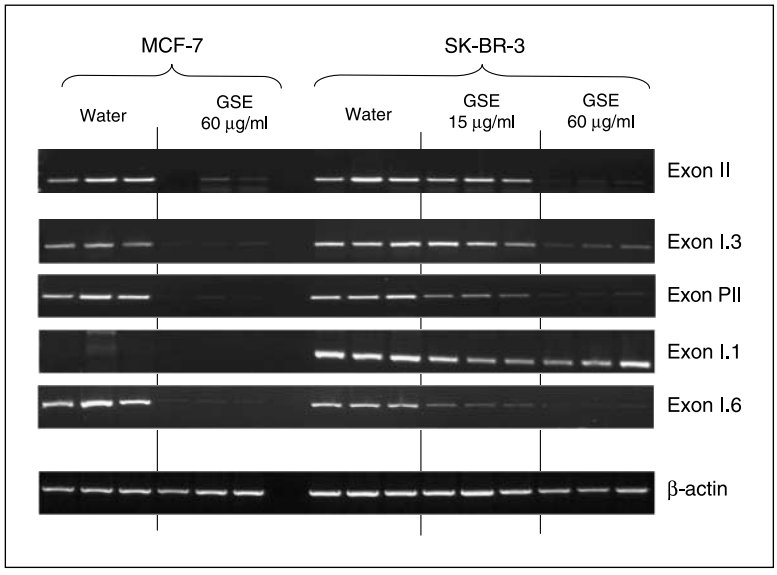

Figure 3. Exon I–specific RT-PCR analysis of GSE-treated MCF-7 and SK-BR-3 cells. MCF-7 and SK-BR-3 cells were treated with 15 or 60 μg/mL of GSE or with water as a control. Treatment and RT-PCR were done in triplicate. After 24 hours of incubation with GSE or water, total RNA was isolated. Ten micrograms of total RNA were used for RT-PCR as described in Materials and Methods. The β-actin was amplified as an internal control. The size of the detected PCR products for exons II, I.3, PII, I.1, and I.6 and β-actin are 169, 333, 234, 256, 1,037, and 825 bp.

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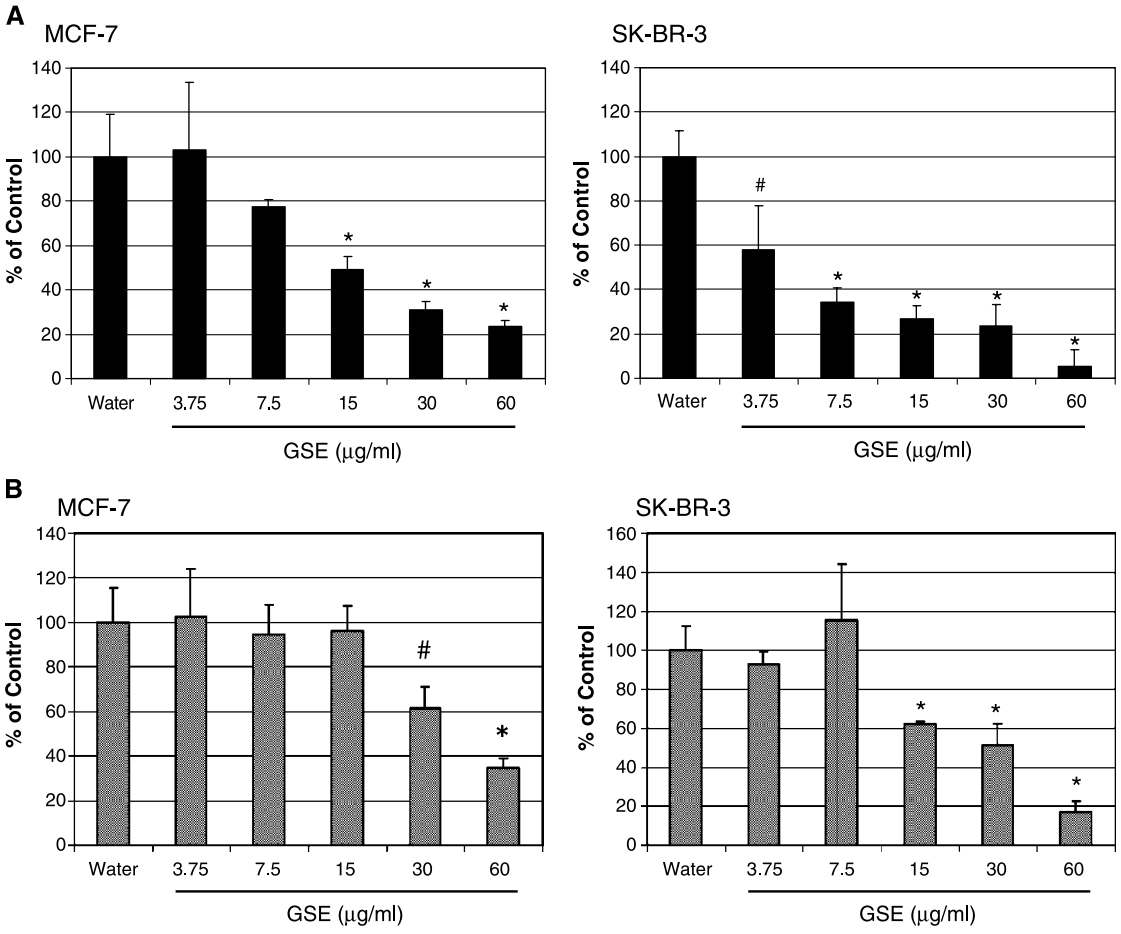

Figure 4. Suppressive effect of GSE on aromatase promoter I.3/II and I.4 activity. MCF-7 and SK-BR-3 cells were transiently transfected with 1 μg of pGL3basic-pI.3/II (contains aromatase promoter I.3/II −329/+284 region; *A*) or pGL3basic-pI.4 (contains aromatase promoter I.4 −1,020/+350 region; *B*). After transfection, cells were incubated with water or increasing amounts of GSE for 24 hours and luciferase activity was then measured. The relative luciferase activity was calculated by dividing the light unit of luciferase activity by protein concentration. *Columns,* percent of control (water-treated sample). *, $P \lesssim 0.01$; #, $P < 0.05$, compared with water-treated control (two-tailed Student's *t* test).

decreased on treatment with 60 μg/mL of GSE to 10.9% in MCF-7 cells and 12.7% in SK-BR-3 cells. Protein expression level of CREB-1 and GR is also decreased on treatment with 60 μg/mL of GSE (Fig. 5*B*). However, the decrease of CREB-1 protein expression level in MCF-7 cells was not as clear as in SK-BR-3 cells. This seems to agree with the results obtained from transient transfection experiments showing that the suppression of promoter I.3/II and I.4 activity in SK-BR-3 cells was stronger than that in MCF-7 cells. The cause of this difference is not currently understood. These results suggested that GSE could suppress the expression of CREB-1 and GR. We also examined the expression levels of other transcription factors, including small heterodimer partner (SHP) and C/EBPD, on GSE treatment and found no difference. Therefore, the suppressive effect of GSE on aromatase expression and promoter activity is thought to be mediated in part by decreased expression of CREB-1 and GR.

Discussion

A great deal of research has revealed that GSE possesses powerful protective properties against free radicals and oxidative stress, and exhibits a wide range of biological advantages such as antibacterial, antiviral, anti-inflammatory, and antiallergic actions. GSE has also been linked to cancer prevention/therapy and exhibits cytotoxicity to cancer cells but not to normal cells (30). Previously, our laboratory showed that procyanidin B dimers purified from red wine are potent inhibitors of aromatase. Because grape seed is a rich source of procyanidine B dimers, we decided to investigate whether GSE would also inhibit aromatase. In this study, we were able to confirm that GSE indeed exhibits inhibition of aromatase activity. More interestingly, this is the first report to show that GSE suppresses not only aromatase enzymatic activity but also aromatase expression and promoter activity.

GSE suppressed promoter I.3/II and I.4 activity in MCF-7 and SK-BR-3 cells. Furthermore, GSE reduced the expression of two transcription factors, CREB-1 and GR, which are known to stimulate promoter I.3/II and I.4 activity, respectively. The existence of exon I.6 was found in THP-1 cells (mononuclear leukemia cell line) and promoter I.6 was characterized by Shozu et al. (35, 36). Exon I.6 is expressed highly in testis, bone, and fetal liver. The expression of exon I.6 is enhanced strongly by serum and phorbol ester in THP-1 and JEG-3 cells (choriocarcinoma

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cell line). Furthermore, in fetal osteoblasts, promoter I.6 is also activated to a lesser extent by dexamethasone, which is a ligand of GR. This raised a possibility that suppression of exon I.6 expression on GSE treatment could be mediated through down-regulation of GR. Exon I.1 is a placenta dominant exon I and also reported to be up-regulated by cAMP and phorbol ester (37, 38). However, we did not see down-regulation of exon I.1 on GSE treatment. Effect of cAMP on exon I.1 could be through an indirect mechanism as suggested by Harada et al. (37) because promoter I.1 does not contain typical CRE motifs.

Grape seed procyanidins have been shown to lower plasma triglycerides, free fatty acids, and lipoproteins. It also induced mRNA levels of liver cholesterol 7α-hydroxylase (CYP7A1), SHP, and cholesterol biosynthetic enzymes in rats (39). SHP is an atypical nuclear receptor that lacks a DNA binding domain and heterodimerizes with other nuclear receptors such as LRH-1, resulting in inhibition of the functions of other nuclear receptors which SHP binds to (40). LRH-1 induces aromatase expression in preadipocytes by binding to a single hexameric element, AGGTCA, within the S1 region of promoter II (41, 42). SHP is reported to inhibit aromatase transcription induced by LRH-1 in 3T3-L1 preadipocytes (43). Therefore, it was thought that suppression of aromatase exon I.3/II expression and their promoter activity by GSE could be mediated by up-regulation of SHP. However, real-time PCR experiments with cDNA obtained from MCF-7 and SK-BR-3 cells treated with 60 μg/mL of GSE showed no significant difference in SHP expression compared with water-treated control (data not shown). Del Bas et al. (39) observed an increased level of SHP on GSE treatment in rat liver whereas we examined the SHP expression in breast cancer epithelial cells. As discussed above, SHP may play important roles in modulating aromatase expression in preadipocytes, but not in breast cancer epithelial cells.

GSE concentrations used to produce the suppressive effects on aromatase activity (40 and 60 μg/mL) and expression (15, 30, and 60 μg/mL) *in vitro* were relatively high when we consider that a 750-μg dose could significantly reduce tumor growth *in vivo*. This may be because GSE exhibits other activities in cells, such as proapoptotic, antiproliferative, and antiangiogenesis activities reported by Agarwal et al. (29, 44, 45), besides just suppressing aromatase activity and expression. In our *in vitro* experiments, we examined GSE effects on aromatase activity and expression only. However, in the mice experiments, we measured tumor reduction, the result of which is subject to the combinatorial effects of GSE, which include but are not limited to suppression of aromatase.

During the last several years, both preclinical (46, 47) and clinical studies (48–50) have shown that aromatase inhibitors (such as letrozole, anastrozole, and exemestane) achieve better results than tamoxifen (an antiestrogen) in treating hormone-dependent breast cancer. However, because aromatase inhibitors are very effective in suppressing estrogen formation, patients have also experienced side effects associated with estrogen deficiency, such as bone loss. Aromatase researchers including us are interested in finding ways to suppress aromatase expression in breast tumors and to design treatment strategies that selectively suppress its expression in breast tumor tissue while also maintaining estrogen levels in normal tissues. Suppression of aromatase by GSE through CREB-1 and GR may not be specific for inhibiting aromatase expression because CREB-1 and GR are also involved in regulation of other

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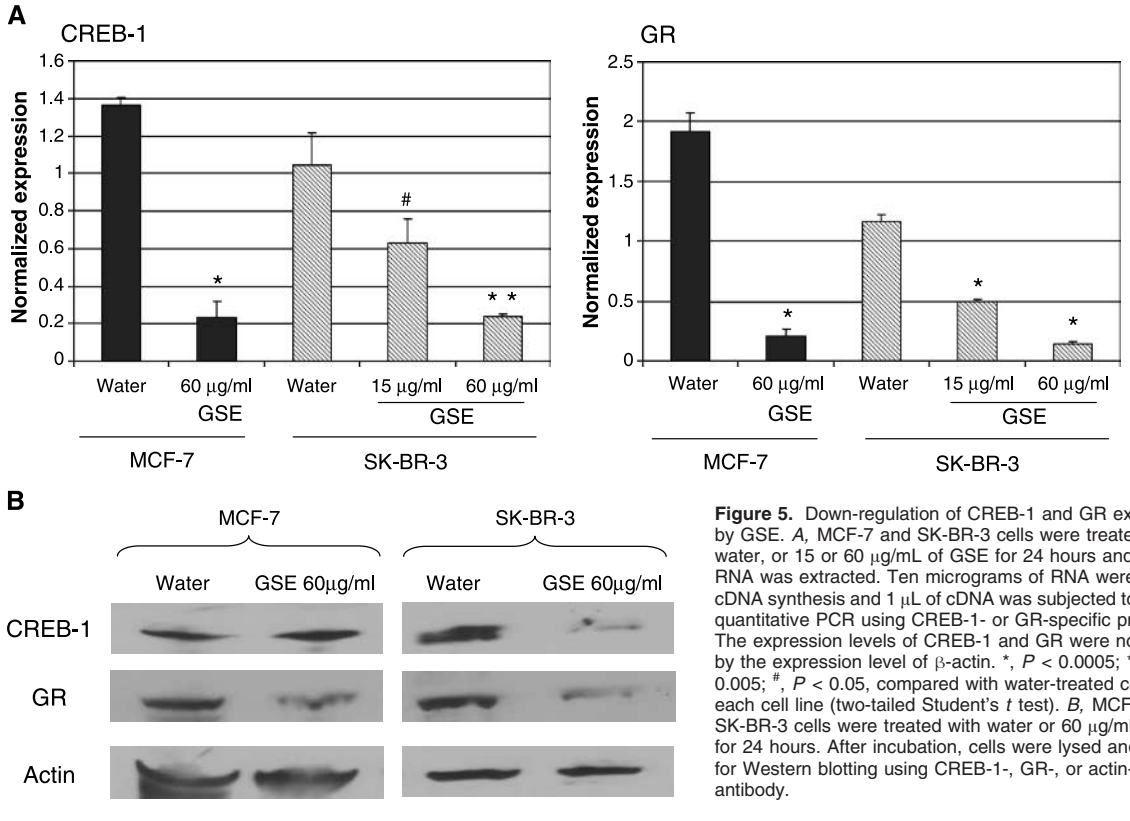

Figure 5. Down-regulation of CREB-1 and GR expression by GSE. *A,* MCF-7 and SK-BR-3 cells were treated with water, or 15 or 60 μg/mL of GSE for 24 hours and total RNA was extracted. Ten micrograms of RNA were used for cDNA synthesis and 1 μL of cDNA was subjected to real-time quantitative PCR using CREB-1- or GR-specific primer. The expression levels of CREB-1 and GR were normalized by the expression level of β-actin. *, $P < 0.0005$; **, $P < 0.005$; #, $P < 0.05$, compared with water-treated control of each cell line (two-tailed Student's *t* test). *B,* MCF-7 and SK-BR-3 cells were treated with water or 60 μg/mL of GSE for 24 hours. After incubation, cells were lysed and applied for Western blotting using CREB-1-, GR-, or actin-specific antibody.

genes besides aromatase. However, it should be noted that GSE does indeed suppress aromatase and it can be used as a preventive agent as shown by the animal experiment. At this moment, we have not yet identified the chemicals in GSE that inhibit aromatase expression. However, our results presented here led us to propose that some unidentified chemical(s) in GSE is able to suppress aromatase expression in breast tumors. Based on this finding, experiments are being carried out in our laboratory to search for the active chemicals that suppress aromatase expression.

In summary, our results indicate that GSE can suppress estrogen production in breast cancer through at least two mechanisms. First, it inhibits the enzymatic activity of aromatase. More interestingly, through suppression of the expression of CREB-1 and GR, GSE will decrease the expression of aromatase in breast cancer tissue by reducing the activity of promoters I.3, II, and I.4. In addition, our animal studies have confirmed that GSE intake suppresses aromatase-expressing breast tumor growth *in vivo*. Based on results of preclinical studies from this and other laboratories, a phase I chemoprevention clinical trial involving GSE in postmenopausal women has been initiated at our institution, the City of Hope.[1] GSE is a common dietary supplement that is widely used. Elucidating the mechanisms of down-regulation of aromatase activity and expression via breast cancer or breast stromal cells-specific promoters by GSE would help in designing prevention strategies that selectively suppress its expression and activity in breast tumor tissue while also maintaining estrogen levels in normal tissues.

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[1] http://clinicaltrials.coh.org/study_display.aspx?pid=3713861.

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