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Gynecologic Oncology 94 (2004) 760–768

Gynecologic Oncology

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# Anticancer effects of (−)-epigallocatechin-3-gallate on ovarian carcinoma cell lines

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Received 5 February 2004
Available online 15 July 2004

**Abstract**

*Purpose.* A constituent of green tea, (−)-epigallocatechin-3-gallate (EGCG), has been known to possess anti-cancer properties. In this study, we investigated the time-course anticancer effects of EGCG on human ovarian cancer cells to provide insights into the molecular-level understanding of growth suppression mechanism involved in EGCG-mediated apoptosis and cell cycle arrest.

*Methods.* Three human ovarian cancer cell lines (p53 negative, SKOV-3 cells; mutant type p53, OVCAR-3 cells; and wild type p53, PA-1 cells) were used. The effect of EGCG treatment was studied via cell count assay, cell cycle analysis, FACS, Western blot, and macroarray assay.

*Results.* EGCG exerts a significant role in suppressing ovarian cancer cell growth. Also, EGCG showed growth inhibitory effects in each cell line in a dose-dependent fashion and induced apoptosis and cell cycle arrest. The cell cycle was arrested at the $G_1$ phase by EGCG in SKOV-3 and OVCAR-3 cells. In contrast, the cell cycle was arrested in the $G_1$/S phase arrest in PA-1 cells. EGCG differentially regulated the expression of genes and proteins (Bax, p21, Retinoblastoma, cyclin D1, CDK4, Bcl-$X_L$) more than 2-fold, showing a possible gene regulatory role of EGCG. The continual expression in p21WAF1 suggests that EGCG acts in the same way with p53 proteins to facilitate apoptosis after EGCG treatment. And Bax, PCNA, and Bcl-X are important in EGCG-mediated apoptosis. In contrast, CDK4 and Rb are not important in ovarian cancer cell growth inhibition.

*Conclusion.* EGCG can inhibit ovarian cancer cell growth through induction of apoptosis and cell cycle arrest as well as regulation of cell cycle-related proteins. Thereby, the EGCG-mediated apoptosis can be applied to an advanced strategy in the development of a potential drug against ovarian cancer.


*Keywords:* (−)-epigallocatechin-3-gallate (EGCG); Ovarian Cancer; Apoptosis; Cell Cycle

## Introduction

Ovarian cancer is a leading cause of death from cancer in women. Without clearly definable symptoms, ovarian cancer often remains undetected until an advanced stage, when prognosis is poor. The main causes are considered to be sporadic in nature and to arise as a result of acquired alterations in oncogenes and tumor suppressor genes such as TP53 and PTEN [1,2]. The alterations that lead to ovarian cancer vary between patients, and the striking clinical heterogeneity of ovarian cancer likely reflects an underlying molecular heterogeneity. To understand the possible causes of heterogeneity of cancer cells, it is recommended that the obvious thing to do is to avoid drugs that may increase heterogeneity by triggering further mutations or anueploidy [3].

Green tea has potentially preventive effects against cancer [4]. Anti-proliferative effects of green tea preparations have been demonstrated in many cancer cell lines and different animal models [5,6]. The anti-carcinogenic and anti-proliferative effects of tea have been attributed to the biological

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doi:10.1016/j.ygyno.2004.05.031

properties of green tea polyphenolic compounds, especially, (−)-epigallocatechin-3-gallate (EGCG) [7,8]. EGCG has demonstrated anti-carcinogenic activities in human and animal models including cancer of the breast, prostate, stomach, esophagus, colon, pancreas, skin, and lung [9–11]. In vitro studies have also shown that EGCG can inhibit growth of human mammary and lung cancer cells, prostate cancer cells, lymphoma cells, leukemic cells, and lung and colon cancer cell lines [12,13]. EGCG can ameliorate free radical damage to DNA under certain conditions [14,15]. However, even though, several molecules have been proposed as a target for EGCG, such as urokinase, matrix metalloproteinases, vascular endothelial growth factor (VEGF), tyrosine kinase receptors, and cell cycle regulators [16–19], the precise mechanisms by which EGCG exerts its anti-carcinogenic activity are still not fully understood. Furthermore, no studies on ovarian cancer cells have been reported.

In this study, we investigated whether a major green tea constituent, EGCG possesses growth inhibitory properties in various human ovarian cancer cell lines. We also observed that EGCG possesses a potent growth inhibitory ability in ovarian cancer cell lines in a dose dependent fashion. This growth inhibition appears to be mediated by apoptosis, cell cycle arrests in the $G_1$ phase, and regulation of gene expression, as determined by FACS and macroarray containing genes involved cell death. This report allows the simultaneous, quantitative measurement of the expression of many gene induced by EGCG, resulting in important understanding how a cell decides to undergo apoptosis or growth arrest.

## Materials and methods

### *Cells*

SKOV-3 (p53 negative), OVCAR-3 (mutant type p53), and PA-1 (wild type p53) cells were obtained from the Korean Cell Line Bank, Seoul National University, Seoul, Korea. All cells were cultured in DMEM (Gibco, Rockville, MD) supplemented with 5% heat-inactivated FBS (Fetal Bovine Serum) (Gibco), 0.37% sodium bicarbonate (Sigma, St. Louis, MO), and streptomycin/penicillin (Gibco) at 37°C in the 5% $CO_2$ incubator.

### *Cell growth inhibition assay*

For the inhibition assay of cell proliferation, EGCG (a kind gift from Dr. Yukihiko Hara of Mutsui Norin Co., Fujied, Japan) was diluted 50% DMSO and stored at −20°C before use. For viable cell counting, $5 \times 10^3$ cells per well were treated with EGCG ranging from 6.25 to 100 μM for various times. Cells were counted using a hemacytometer under a microscope after trypsinization. Cell viability was determined by trypan blue exclusion assay. From analysis of three replicates, the typical measurement deviations were observed to be less than ±3.0% for each assay.

### *DNA fragmentation assay*

Each cell lines were divided into $5 \times 10^5$ cells/100 mm dish plate. After 24-h incubation, cells were added with different amounts of EGCG. After 24 or 48 h, the cells were centrifuged and subsequent cell pellets were added with lysis buffer [0.5% SDS, 0.1 M NaCl, 0.01 M EDTA, 10 mM Tris–HCl (pH 8.0)], followed by addition of 20 μg/ml proteinase K and 10 mg/ml RNase A (Sigma). This was incubated for 4 h at 56°C. DNA was extracted by phenol/chloroform treatment. Five micrograms of extracted DNA was analyzed on a 2% agarose gel containing ethidium bromide (EtBr) (0.1 μg/ml). DNA ladder formation was visualized under UV light.

### *Cell cycle analysis*

Cell suspensions were treated with several concentrations of EGCG for 1 and 2 days. The cells were then trypsinized and washed twice with phosphate-buffered saline (PBS). Cells were stained with propidium iodide (100 μg/ml in PBS). Samples were then analyzed using FACS (Becton Dickinson, San Jose, CA). For DNA contents, cell debris and fixation artifacts were gated out and $G_0/G_1$, S, and $G_2/M$ populations were quantified using the CellQuest program.

### *FACS analysis*

Cell suspensions were centrifuged and resuspended in phosphate-buffered saline (PBS) to a concentration of $5 \times 10^5$ cells/100 mm dish. After EGCG treatments with several concentrations, cell were incubated with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI) in the dark at room temperature for 10 min followed by fixation with 2% formaldehyde. The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (Becton Dickinson, San Jose, CA). The forward and side scatter gates were set to exclude any dead cells from the analysis. At least 10000 events were collected for each sample.

### *Membrane chip analysis*

Total cellular RNA of SKOV-3 was isolated with acid-guanidinium-thiocyanate-phenol chloroform method using the Tri-Reagent (Molecular Research, Inc., Cincinnati, OH). The apoptosis pathway GEArray Q Series Human Cell Cycle Gene Array http://www.superarray.com) was obtained from SuperArray Inc. (Bethesda, MD). This kit determines differential expression levels of multiple genes involved in a biological pathway. Briefly, the total RNA from the respective samples was used as a template to generate cDNA probes using the GEAprimer mix as a reverse transcriptase primer. The cDNA probes, which represent abundance of mRNA population, were then denatured and hybridization was conducted in GEHybridization solution to two nylon

membranes spotted with gene-specific cDNA fragments. Membranes were then washed in 2 × SSC, 1% SDS twice for 15 min each followed by 0.1 × SSC, 0.5% SDS twice for 15 min each. The membranes were then exposed to ChemiDoc (BioRad Laboratories, Hercules, CA). The relative expression level of each gene was then determined through GEArray Analyzer program (Superarray). Measurements were taken on two separated arrays.

*RT-PCR*

Total RNA was isolated from cells using Tri-reagent (Gibco) and used as templates. Reverse transcription was carried out using RNase (Invitrogen, Carlsbad, CA), and performed at 22°C for 10 min and then at 42°C for 20 min using 1.0 μg of RNA per reaction. The primers used in the PCR reactions for the genes analyzed are summarized in Table 1. PCR was performed on the EGCG-treated cell lines under 35 cycles at 94°C for 1 min, at 57°C for 1 min, and at 72°C for 1 min. The amplified products were subjected to electrophoresis on a 2% agarose gel. The gel was then stained with EtBr and photographed. The specific primers (forward: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, reverse: 5′-CTAGAAGCATTT-GCGGTGGACGGATG-GAGGG-3′) were used for control gene β-actin. The amplification reaction involved denaturation at 94°C for 30 s and annealing at 72°C for 30 s, and 72°C for 45 s for 20 cycles.

*Western blot*

The cells grown in 6-well culture dishes ($5 \times 10^5$) were treated with several concentrations of EGCG. After 24 or 48 h, the cells were lysed. The protein concentration of the clear supernatant collected by centrifugation was evaluated by using the BioRad Protein-Assay kit (Bio-Rad, Hercules, CA) and was adjusted to give the final concentration of 2 mg/ml. After addition of 2-mercaptoethanol (2%), samples were boiled for 5 min and used for the experiments. The 40 μg of each protein sample underwent electrophoresis for 2 h with SDS-PAGE at 10 mA, and the blotting was performed with a Hybond-ECL membrane (Amersham, Uppsala, Sweden) at 100 V. The blotted membrane was blocked with 5% skim milk and reacted with a primary polyclonal antibody (rabbit CDK2, E2F-1, E2F-4, CDK4; Santa Cruz Biotechnology Inc., Santa Cruz, Ca) and a primary monoclonal antibody (mouse P21, Bax, Rb, Cyclin D1, BclXl, PCNA, Actin; Pharmingen, Franklin Lakes, NJ) at 1 mg/ml for 4 h at room temperature. After washing with Tris-buffered saline containing 0.1% Tween 20, the membrane was then incubated with the horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch, Bar Harbor, MA) at a dilution of 1:1000. Protein bands were visualized using an ECL Kit (Amersham, Arlington Heights, IL).

Table 1
List of primers and product sizes for RT-PCR

| Type | Primer sequence | Length |
|---|---|---|
| p57 | 5′ CCT CCG CAG CAC ATC CA 3′ | 696 |
| | 5′ GCA GCT GGT CAG CGA GA 3′ | |
| Apaf-1 | 5′ CTC AAC AGC AAA GAG CA 3′ | 700 |
| | 5′ CCT AAG GAA CTC TCC ACA 3′ | |
| RAD9 | 5′ CCC GCT CTT CTT CCA GCA 3′ | 698 |
| | 5′ GTG TGC TGT GAG CCT GGA 3′ | |
| p130 | 5′ GCA TTT CCA GCC AGA CGA 3′ | 699 |
| | 5′ TTC TGC ACA GGG ACA GCA 3′ | |
| CDC2 | 5′ GGG GTT CCT AGT ACT GCA 3′ | 531 |
| | 5′ CAG TGC CAT TTT GCC AGA 3′ | |
| MCM-7 | 5′ AGG ATT GCC CAG CCT GGA 3′ | 800 |
| | 5′ TGG GCC AAC CGT AGG TCA 3′ | |

*Statistical analysis*

Statistical analysis was done using ANOVA. Values between different groups were compared. *P* values of less than 0.05 were considered significant. All the data shown in the study for cell cycle analysis, and cell growth inhibition are representative of three independent studies.

## Results

*EGCG inhibits ovarian cancer cell growth in a dose-dependent manner*

To determine anti-tumor effects of EGCG in three different ovarian cancer cell lines, such as SKOV-3, OVCAR-3 cells, and PA-1, we treated cells with EGCG ranging from 10 to 100 μM. As shown in Fig. 1, EGCG showed significant growth inhibitory effects in these ovarian cancer cell lines over the treatment time. The inhibition of cell growth was also dose-dependent.

*EGCG induced cell cycle arrests in ovarian cancer cells*

We also tested for cell cycle arrests in ovarian cancer cells. To determine if EGCG treatment has any effects on cell cycle perturbations, cell cycle analysis was performed by propidium iodide staining and flow cytometric analysis. As shown in Table 2, the percent distribution of cells in different cell cycle phases showed that after treatment with EGCG, a significant alteration in cell cycle progression in each cell line was shown, which suggests that EGCG induces the cell growth arrest in ovarian cancer cells. In the control cells, the percent distribution of PA-1 in different cell cycle phases showed a difference as compared to other cell lines after 24 to 48 h of culture. When treated with each concentration of EGCG, the percentage of SKOV-3 cells in the $G_1$ phase was significantly increased for 48 h, which suggests that the cell cycle was arrested at the $G_1$ phase. The $G_1$ phase arrest profiles of EGCG were consistent over the time points in OVCAR-3 cells, where the G1 phase are significantly in-

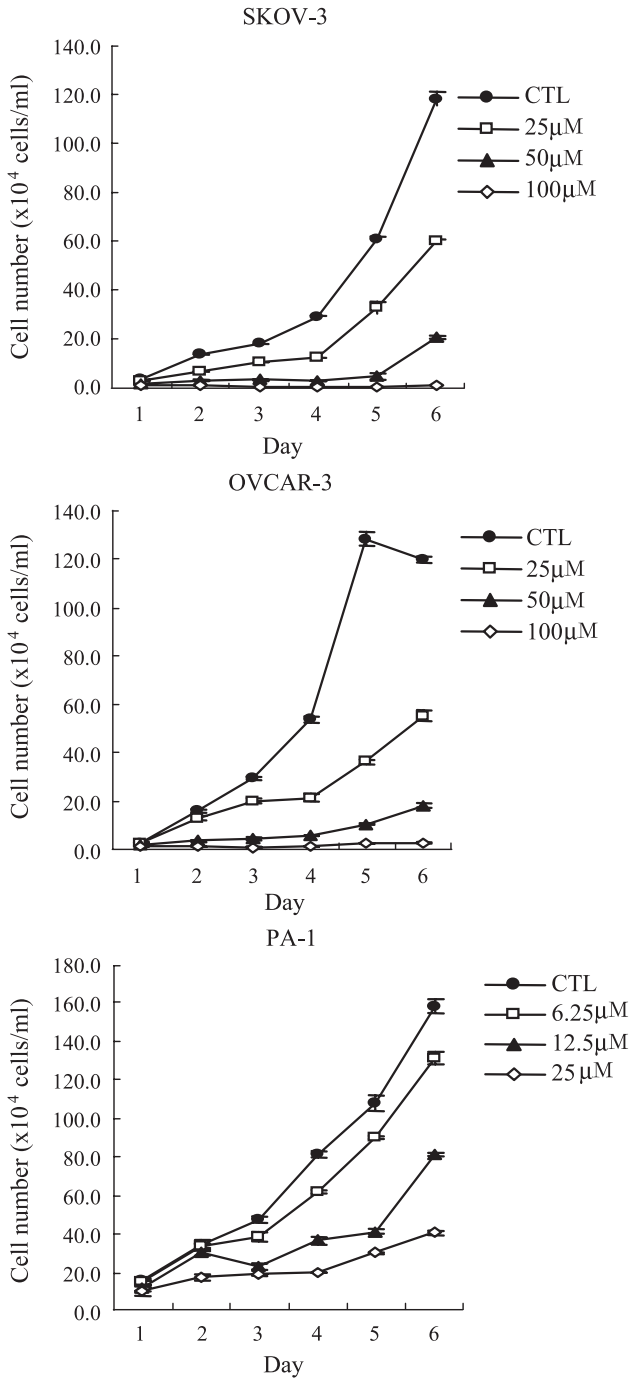


Fig. 1. Growth inhibition effects of EGCG on various ovarian cancer cell lines, SKOV-3, OVCAR-3, and PA-1 cells at different concentrations. Cells ($10^5$ cells/well) were cultured in 12-well plates in triplicate overnight and treated with EGCG at increasing concentration(s). After EGCG treatment, cells were cultured for 6 days and then trypsinized for counting under a microscope. The mean values of cell counts in triplicate were plotted. Values and bars represent mean and SD, respectively.

creased for 48 h, except that a marked increase in the cell population in $G_2$/M was observed for 48 h. EGCG treatment resulted in a dramatic increase in the $G_1$ cell population even for 24 h. In contrast, there was a significant increase in the cell population of the $G_2$/M phase for 24 h, and S phase for 48 h in PA-1 cells with a compensatory decrease in the population in phases S or $G_2$/M. Especially, after EGCG treatment, cell population in the S phase was significantly increased for 48 h, as compared to other cell lines. These data suggest that EGCG treatment induces cell cycle arrest in $G_1$ phase in SKOV-3 and OVCAR-3 cells, and the time-dependent shift of cell population form $G_2$/M to S phase in PA-1 cells.

### *Cell death induced by EGCG*

The EGCG treatment has been shown to mediate cell death in ovarian cell lines. Uptake of EGCG prevents cells from undergoing proliferation and increases the efficacy of antitumor effect in vitro. To check whether the cell death was accompanied with the development of an apoptotic or necrotic process, we analyzed and quantified the phenotypic changes characteristic of apoptotic cells by double staining ovarian cancer cells with annexin V and propidium iodide (PI). Exponentially growing control cells were exposed to EGCG treatment, and then harvested for the predetermined times. Fig. 2 shows that the cell death increased significantly after EGCG treatment in SKOV-3. That is, a greater induction of cell death (late apoptosis or necrosis) conse-

Table 2
Summary of cell cycle arrest in ovarian cancer cells

| | | $G_0/G_1$ | S | $G_2$/M |
|---|---|---|---|---|
| *SKOV-3* | | | | |
| 24 h | Control | 53.6 ± 0.5 | 38.6 ± 0.3 | 7.6 ± 0.4 |
| | 25 μM | 53.7 ± 0.3 | 38.1 ± 0.4 | 8.0 ± 0.2 |
| | 50 μM | 57.7 ± 0.8 | 37.8 ± 0.3 | 4.4 ± 0.4* |
| | 100 μM | 63.4 ± 0.8* | 21.6 ± 0.9* | 14.9 ± 0.4* |
| 48 h | Control | 54.6 ± 0.3 | 27.2 ± 0.1 | 9.7 ± 0.3 |
| | 25 μM | 62.7 ± 0.9 | 31.6 ± 0.9* | 5.6 ± 0.6* |
| | 50 μM | 63.6 ± 0.3* | 29.8 ± 0.8* | 6.5 ± 0.2* |
| | 100 μM | 61.9 ± 0.9 | 26.7 ± 0.4 | 11.3 ± 0.5* |
| *OVCAR-3* | | | | |
| 24 h | Control | 51.3 ± 0.7 | 40.3 ± 0.2 | 8.3 ± 0.3 |
| | 25 μM | 70.7 ± 1.1* | 22.4 ± 0.2* | 6.7 ± 0.7 |
| | 50 μM | 73.8 ± 0.4* | 16.6 ± 0.4* | 9.5 ± 0.8 |
| | 100 μM | 59.8 ± 0.4 | 16.7 ± 0.3* | 23.4 ± 0.5* |
| 48 h | Control | 47.1 ± 0.5 | 52.8 ± 0.4 | 0.1 ± 0.3 |
| | 25 μM | 67.1 ± 0.4* | 22.3 ± 0.5 | 10.5 ± 0.3* |
| | 50 μM | 69.1 ± 0.8* | 13.1 ± 0.8* | 17.6 ± 0.7* |
| | 100 μM | 65.1 ± 0.3 | 19.0 ± 0.9* | 15.7 ± 0.7* |
| *PA-1* | | | | |
| 24 h | Control | 32.8 ± 0.3 | 49.4 ± 0.5 | 17.6 ± 0.7 |
| | 25 μM | 33.6 ± 0.2 | 47.8 ± 0.4 | 18.5 ± 0.6 |
| | 50 μM | 33.0 ± 0.9 | 41.3 ± 0.8 | 25.6 ± 0.4* |
| | 100 μM | 33.4 ± 0.3 | 40.1 ± 0.3* | 26.4 ± 0.5* |
| 48 h | Control | 34.3 ± 0.5 | 41.3 ± 0.4 | 19.6 ± 0.6 |
| | 25 μM | 33.8 ± 0.6 | 39.8 ± 0.2 | 26.3 ± 0.8* |
| | 50 μM | 33.3 ± 0.9 | 47.3 ± 0.3 | 19.3 ± 0.6 |
| | 100 μM | 32.7 ± 0.2 | 51.1 ± 0.7* | 16.0 ± 0.4 |

Cells were cultured as described in Materials and methods and treated with EGCG at increasing concentration(s) for 24 and 48 h. The data shown are mean + standard deviation of three independent plates.
* $P < 0.05$ for differences with control group.

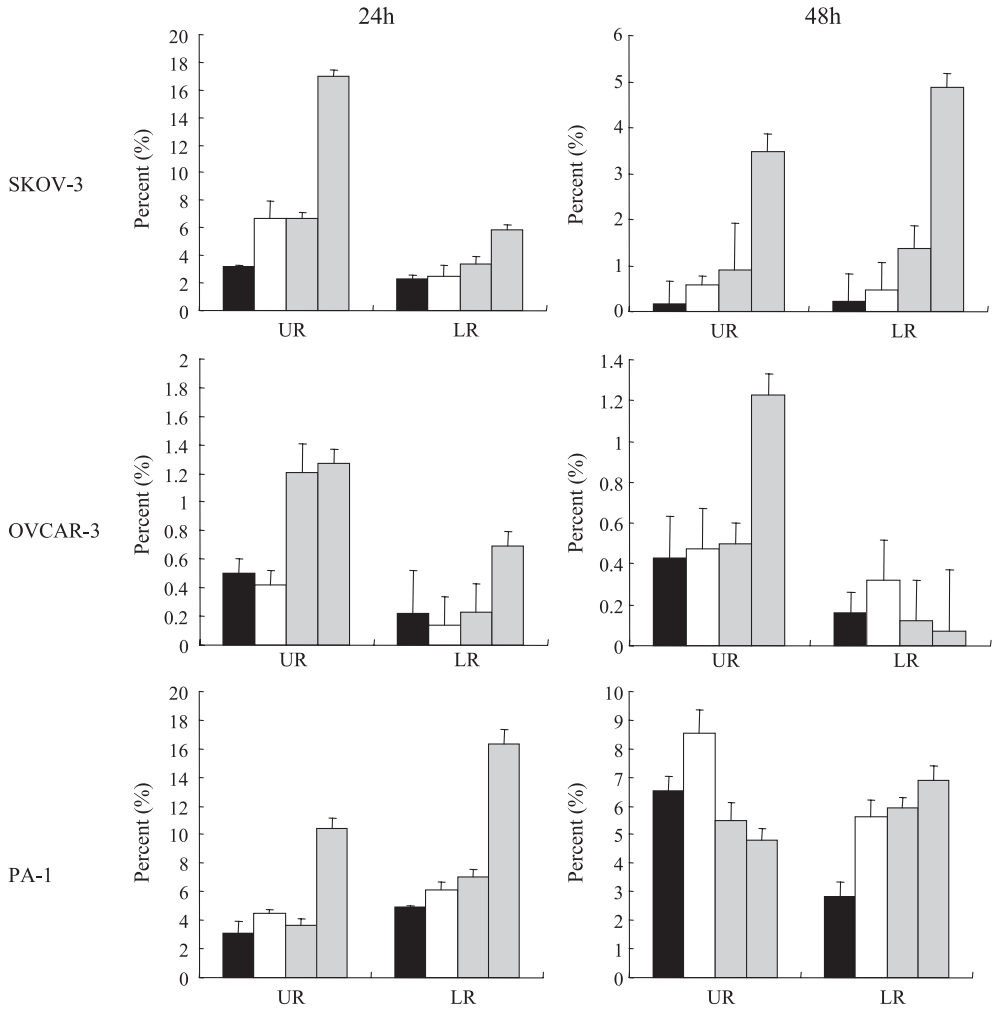


Fig. 2. Detection of EGCG-induced cell death in ovarian cancer cells. Quantitative analysis of the apoptotic cells using annexin V-FITC and PI in exponentially growing SKOV-3, OVCAR-3, and PA-1 cells Each cell line was incubated for 2 days. The flow cytometric analysis was performed on $10^5$ cells and the percentage of apoptotic, live, and dead cells were measured. The graph shows that the percentage of lower right (LR; annexin$^+$/PI$^-$) cells represents early apoptotic cells, and the upper right (UR; annexin$^+$/PI$^+$) dead (late apoptotic plus necrosis) cells. Data are the mean and SD and representative of three separate experiments. Quadrant statistics for labeled cells are displayed for ■ control, □ 25 μmol, ▒ 50 μmol, and ▩ 100 μmol.

quent upon EGCG uptake was observed in SKOV-3 cells for 24 h at every EGCG concentration, suggesting later stages of apoptosis and cells undergoing rapid secondary necrosis in culture. The cell death rates increased sharply during the first day and showed a slower decrease thereafter, changing the cell death patterns into early apoptosis for 48 h after EGCG treatment. On the other hand, lower sensitivity to EGCG was shown in OVCAR-3 cells as compared to SKOV-3 cells, besides, a distinct population of early apoptotic cells was not shown. Early apoptotic populations of cells were observed in SKOV-3 and PA-1 cell lines. In contrast, PA-1 cells underwent early apoptosis for 48 h. The cells exhibited a much higher susceptibility to early apoptosis in response to EGCG treatment in comparison to later apoptosis or necrosis. As expected, there was no detectable cell death effect with control cells. These results showed that EGCG induced apoptotic cell death in each ovarian cancer cell line.

*EGCG controlled the expression levels of several transcripts for 24 and 48 h*

To determine the roles of molecules responsible for EGCG function, changes in the levels of transcripts regulated by EGCG were studied by macroarray analyses after ovarian cancer cells were treated with EGCG. In SKOV-3 cells, several transcripts were upregulated or downregulated after EGCG uptake. Especially, the expression profiles were more prominent for 48 h than the ones for 24 h. The transcripts at least 2-fold up- or down-regulated commonly by EGCG over the time points (24, 48 h) are listed in Table 3. As shown in Tables 3 and 4, the expression of several transcripts was down-regulated more than 2-fold in 48 h treatment with EGCG. The p55cdc, cyclin B1, and cyclin A transcripts levels were found to significantly decrease at 48 h post-EGCG uptake. In particular, during this period, a 43.4-fold decrease in p55cdc was detected, suggesting the most sensi-

tivity to EGCG treatment. However, only BCL2-associated X protein was found to be highly upregulated for 48 h, which showed a 2.7-fold decrease. In contrast, the expressions of 12 transcripts were down-regulated more than 2 fold by 24 h treatment with EGCG. Among them, the transcripts encoding for E2F transcription factor 1 and DP-1 were found to be significantly downregulated by EGCG for 24 h. The cullin 3, E2F transcription factor 2, cyclin B1, and CDC28 protein kinase 2 were also shown to be downregulated for 24 h. In contrast, the cell division cycle 2 and $G_1$/S-specific cyclin C were only shown to be upregulated for 24 h. These data suggest that EGCG can influence a gene expression pattern in SKOV-3 cells. Furthermore, it is likely that these regulated genes might be possibly associated with resistance/sensitivity to EGCG in this cell line.

### *EGCG controlled the expression levels of several proteins for 24 and 48 h*

Northern and Western blot analyses of several proteins associated with growth arrest and/or apoptosis have been performed with SKOV-3 cells uptaken by EGCG at 25 μM for 24 and 48 h. As shown in Fig. 3, several up- and down-regulated transcripts confirmed the patterns obtained from the macroarray, showing the consistency of our experiment.

Table 3
Summary of gene expression changes for 24 h

| Unigene no. | Description | Fold change |
|---|---|---|
| *Upregulated genes* | | |
| Hs.184572 | Cell division cycle 2, G1 to S and G2 to M | 2.34 |
| Hs.118442 | G1/S-specific cyclin C | 2.11 |
| *Downregulated genes* | | |
| Hs.79353 | Homo sapiens E2F-related transcription factor (DP-1) | −3.20 |
| Hs.96055 | E2F transcription factor 1 | −2.28 |
| Hs.155462 | Minichromosome maintenance deficient (mis5, *S. pombe*) 6 | −2.19 |
| Hs.77171 | Minichromosome maintenance deficient (*S. cerevisiae*) 5 | −2.12 |
| Hs.4854 | Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) | −2.09 |
| Hs.179565 | Minichromosome maintenance deficient (*S. cerevisiae*) 3 | −2.08 |
| Hs.77152 | Minichromosome maintenance deficient (*S. cerevisiae*) 7 | −2.05 |
| Hs.83758 | CDC28 protein kinase 2 | −2.05 |
| Hs.23960 | Cyclin B1 | −2.04 |
| Hs.121487 | E2F transcription factor 2 | −2.03 |
| Hs.78946 | Cullin 3 | −2.00 |
| Hs.57101 | Minichromosome maintenance deficient (*S. cerevisiae*) 2 | −2.00 |

The genes are ranked by fold change from up-regulation to down-regulation.
The intensity of the digitalized signals was measured using GEArray Analyzer program (Superarray). Signal intensities were standardized to the signals of the housekeeping genes beta-actin.

Table 4
Summary of gene expression changes for 48 h

| Unigene no. | Description | Fold change |
|---|---|---|
| *Upregulated genes* | | |
| Hs.159428 | BCL2-associated X protein | 2.71 |
| Hs.79101 | Cyclin G1 | 2.01 |
| *Downregulated genes* | | |
| Hs.82906 | p55cdc | −43.42 |
| Hs.23960 | Cyclin B1 | −16.68 |
| Hs.85137 | Cyclin A | −14.19 |
| Hs.184572 | Cell division cycle 2, G1 to S and G2 to M | −3.69 |
| Hs.76932 | Ubiquitin-conjugating enzyme, cell division cycle 34 | −2.82 |
| Hs.95577 | Cyclin-dependent kinase 4 | −2.22 |
| Hs.81424 | Human ubiquitin-related protein SUMO-1 mRNA | −2.05 |
| Hs.14541 | Cullin 1 | −2.00 |

The genes are ranked by fold change from up-regulation to down-regulation.
The intensity of the digitalized signals was measured using GEArray Analyzer program (Superarray). Signal intensities were standardized to the signals of the housekeeping genes beta-actin.

Several of differentially expressed transcripts such as p21, Bax, and E2F-4 were reported to be similarly expressed with protein expressions, respectively. As shown in Fig. 4, human β-actin analysis showed that the amount of protein applied to each lane was essentially the same. Moreover, only one protein band was detected in each lane suggesting that there was no degradation of β-actin. P21 protein was highly expressed after treatment. Bax was found to be upregulated by EGCG. BCL-$X_L$ and proliferating cell nuclear antigen (PCNA) reached its same level at 24 h, and started to significantly decrease at 48 h. CDK2 band was observed, but this band was slightly weaker compared with other proteins bands. Although both CDK4 and cyclin D1 were detected, the levels of these proteins were found to remain unchanged upon the EGCG treatment.

## Discussion

A variety of the chemotherapeutical agents, currently in use for therapy against ovarian cancers, are known to cause cytotoxicity to normal cells. Also, acquired resistance to chemotherapy is an important factor resulting in the high mortality of ovarian cancer. In this regard, it has been regarded of significance to develop drugs that provide specific cell death to cancer cells. The growth inhibitory effects of green tea have been reported in many tumor cell lines [8,20–24]. However, no studies have been reported on the effects of individual green tea components in ovarian cancer cells and no studies that have been able to link these in vitro findings with changes at the DNA level. Our observation demonstrated that a major green tea component, EGCG, possesses growth inhibitory activities against ovar-

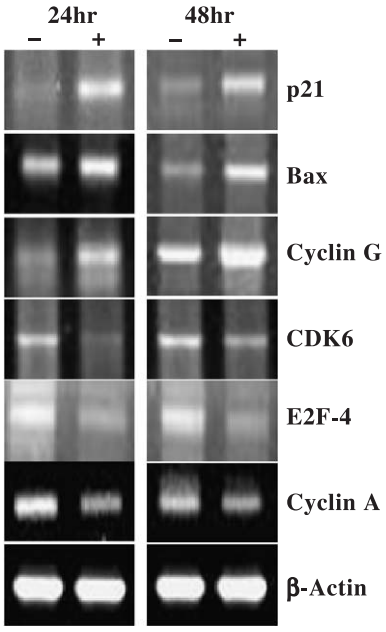


Fig. 3. Confirmation of macroarray assay by RT-PCR analysis. Total RNA obtained from SKOV-3 cells without EGCG treatment (lane – ) and with EGCG treatment (lane+) was subjected to RT-PCR analysis as described in Materials and methods.

ian cancer cell lines. This is consistent with the previous reports in other cancer cell lines, showing that EGCG is the most effective green tea component for inhibition of ovarian cancer cell growth.

Despite chromosomal alterations findings, there are currently few examples of specific cytogenetic studies that have contributed to the clinical management of solid tumors such as ovarian cancer [25]. The main causes of the cancer are considered to be sporadic in nature and to arise as a result of acquired alterations in oncogenes and tumor suppressor genes such as p53 and PTEN [1,2]. The alterations that lead to ovarian cancer vary between patients, and the striking clinical heterogeneity of ovarian cancer likely reflects an underlying molecular heterogeneity. Thus, understanding the molecular basis of anti-cancer effect of EGCG on ovarian cancer progression will be strongly dependent on advanced molecular cytogenetics and genomic profiling methods.

In this study, we observed a significant growth suppression of ovarian cancer cells when EGCG was delivered into the cells, as determined by annexin V staining. In these cells, differentially expressed genes may induce cell cycle arrest and then promote cell growth inhibition as shown in the macroarray and Western blot analyses. The results suggest that EGCG regulates the specific gene and protein expression that might be associated with resistance/sensitivity to EGCG in this SKOV-3 cell line. The anti-cancer effect has taken place via EGCG-mediated apoptosis, regardless of its concentration. In contrast, it was reported that anti-cancer effects of adenoviral p53 have taken place via a pathway leading to apoptosis at higher levels of p53 expression, otherwise, through a pathway leading to growth arrest at lower levels of p53 expression [26]. It could be speculated that the possible application of green tea compounds for inhibiting ovarian cancer growth might be dependent upon the effective EGCG concentrations delivered into cancer cells in vivo.

Anti-tumor effects of EGCG have been reported to be mediated by apoptosis [8,13,21,27], inactivation of transcription factors [23], inhibition of urokinase enzyme activities [28], suppression of lipooxygenase and cyclooxygenase activities [29], and $G_1$ arrest of the cell cycle [30]. EGCG has also been reported to be incorporated into the phospholipid bilayer membrane, leading to blocking the tumor promoters from binding to their receptors within the cell membrane as a possible mechanism of EGCG for tumor growth inhibition [20,23]. Kennedy et al. [27] recently reported that green tea extract causes cytochrome *c* release and caspase activation for induction of apoptosis. More recently, it has been reported that EGCG inhibits the activity of topoisomerase I, which plays a critical role in DNA replication, transcription, and chromosome condensation [31]. EGCG also inhibits tumor cell growth by suppressing vascular endothelial growth factor (VEGF)

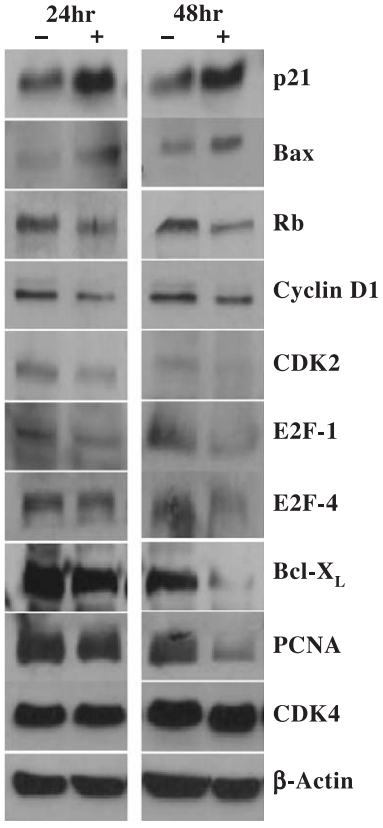


Fig. 4. Western blot analyses for cell cycle-related proteins. Total extracts obtained from SKOV-3 cells without EGCG treatment (lane – ) and with EGCG treatment (lane+) were subjected to Western blot analysis as described in Materials and methods.

induction in human colon cancer cells [32]. Taken together, it is likely that EGCG inhibits cancer cell growth through many different regulatory pathways, along with apoptosis and cell cycle arrests. Our observation also confirmed that EGCG induced apoptosis and cell cycle arrests in the $G_1$ phase in SKOV-3 and OVCAR-3 cell lines, and $G_1$/S phase arrest in PA-1 cells, supporting the previous finding that EGCG arrests cell cycle progression in the $G_1$ phase [8].

In our study, however, cell cycle arrests and apoptosis were dependent upon the ovarian cancer cell lines, supporting the notion that there could be something specific to the molecular-level environment of cell-specific growth suppression effects by EGCG. In SKOV-3 cells, EGCG induces cell cycle arrest in the $G_1$ phase and apoptosis through induction of the expression of p21WAF1, which accords well with our results in the mRNA level, and in the protein level. This result suggests that EGCG acts in the same way as adenoviral p53 proteins do in the HPV-infected cervical cancer cell lines [33].

EGCG induces cell cycle arrests and apoptosis through induction of the expression of Bax in the mRNA level and in the protein level. Bax associates in vivo with Bcl-2 for induction of apoptosis. When Bax predominates, programmed cell death is accelerated, and the death repressor activity of Bcl-2 is countered. Bax-deficient mice indicated that the pro-apoptotic Bax molecule can function as a tumor suppressor [34]. Although Bax is known as a primary-response gene for p53 and is involved in a p53-regulated pathway for induction of apoptosis, it has previously been shown to not be absolutely necessary for p53-dependent apoptosis. Bax mRNA and/or protein levels are not increased after p53 induction [35]. That is, EGCG inhibited the growth of all three ovarian cancer cell lines with different p53 status (p53 negative, SKOV-3 cells; mutant type p53, OVCAR-3 cells; and wild type p53, PA-1 cells), suggesting p53-related pathway is involved. The BCL2 family of proteins, whose members may be antiapoptotic or proapoptotic, binds to the VDAC (Voltage-dependent anion channel) to regulate the mitochondrial membrane potential and the release of cytochrome *c* during apoptosis. In this study, Bax was induced by EGCG in SKOV-3 cells. And Bcl-$X_L$ protein was significantly downregulated at 48 h post-EGCG treatment. It seems that as the ratio of Bcl-$X_L$ to Bax determines survival or death following an apoptotic stimulus [36], Bax and Bcl-$X_L$ are important in EGCG-dependent apoptosis in SKOV-3 cells.

The level of CDK4 was continually found to be expressed in control and SKOV-3 cells after EGCG treatment. CDK4 is activated early in G1 and blocks active repression by Rb through phosphorylation of the C-terminal region of Rb [37]. Thus, CDK4 is not important in EGCG-dependent apoptosis in SKOV-3 cells. On the other hand, EGCG downregulates Rb in the protein level for 48 h. The Rb has been reported to be able to control $G_1$/S transition and function as transcription suppressor [38]. The Rb protein can bind normally to the transcription factors of the E2F family, until it is phosphorylated by the CDK4/cyclin D complex at the restriction point in late $G_1$ phase. Phosphorylated pRb is unable to interact with E2F, therefore, the non-sequestered transcription factor can permit the $G_1$/S transition. As an EGCG-dependent pathway, it seems that there is no connection between EGCG and Rb. In this study, Rb protein was lowered upon EGCG treatment.

Proliferating cell nuclear antigen (PCNA) is a 36-kDa, acidic, non-histone, nuclear protein whose expression is associated with the late G1 and S phases of the cell cycle. PCNA can be used as an immunohistochemical marker of proliferating cells [39]. In mammalian cells, E2F-binding sites are found in promoters of many genes specifically expressed during the S phase and include genes involved in DNA replication such as PCNA. The level of PCNA has been shown to correlate with the proliferative activity and prognosis of patients with head and neck tumors. The level of PCNA was significantly downregulated in SKOV-3 cells after EGCG treatment. Thus, PCNA is important in EGCG-dependent apoptosis in SKOV-3 cells.

In sum, we observed that EGCG treatment exerts an important role in suppressing ovarian cancer cell growth through apoptosis and cell cycle arrest. The mechanism whereby EGCG inhibits ovarian cancer cells growth is related to $G_1$ arrest or G1/S arrest in the cell cycle. These findings show that the continual expression in p21WAF1 suggest that EGCG might act in the same way with p53 to facilitate apoptosis after EGCG treatment. That is, EGCG inhibited the growth of all three ovarian cancer cell lines with different p53 status, suggesting p53-related pathway is involved. Also, Bax and Bcl-X are important in EGCG-dependent apoptosis. For successful medical treatment of ovarian cancer, the molecular-level understanding of cell-specific growth suppression mechanism by EGCG should be further studied in in vivo animal models.

## Acknowledgments

This study was supported by a grant from the Ministry of Agriculture and Forestry (202013-03-1-SB010), Republic of Korea.

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