Epigallocatechin-3-gallate induces apoptosis in estrogen receptor–negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation

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Abstract
Epigallocatechin-3-gallate (EGCG) has been shown to have anticarcinogenic effects in in vitro and in vivo models, and this effect is mediated at least in part by its ability to induce apoptosis in cancer cells without affecting normal cells. It has been recognized that estrogen receptor (ER)–dependent breast cancers generally have a better prognosis and are often responsive to antiestrogen therapy; however, ER-independent breast cancers are more aggressive and unresponsive to antiestrogens. Using the MDA-MB-468 human breast cancer cell line as an in vitro model of ER-negative breast cancers, we found that treatment of EGCG resulted in dose-dependent (5-80 µg/mL) and time-dependent (24-72 hours) inhibition of cellular proliferation (15-100%) and cell viability (3-78%) in MDA-MB-468 cells. Decrease in cell viability was associated with the induction of apoptosis (18-66%) which was analyzed by DNA ladder assay, fluorescence staining, and flow cytometry. Induction of apoptosis by EGCG could be corroborated to the increased expression of tumor suppressor protein p53 and its phosphorylation at Ser 15 residue. EGCG decreased the expression of antiapoptotic protein Bcl-2 but increased proapoptotic protein Bax in these cells. The increased ratio of Bax/Bcl-2 proteins after EGCG treatment may have resulted in increased release of cytochrome c from mitochondria into cytosols, increased expression of Apaf-1, and activation of caspase-3 and poly(ADP-ribose) polymerase, which may lead to apoptosis in MDA-MB-468 cells. Together, the results of this study provide evidence that EGCG possesses anticarcinogenic effect against ER-negative breast cancer cells and thus provide the molecular basis for the future development of EGCG as a novel and pharmacologically safe chemopreventive agent for breast cancer prevention. [Mol Cancer Ther 2005;4(1):81–90]

Introduction
Breast cancer is the most commonly diagnosed invasive malignancy in women affecting as many as one in every eight females. It had been predicted that ~212,600 new cases of invasive breast cancer would be diagnosed and nearly 40,000 women would die from this disease in the year 2003 in the United States alone (1). The diagnosis of breast cancer falls into two broad categories, either estrogen receptor (ER) positive or ER negative, based on the level of the ER in the cancer cells (2). Current therapies for the treatment of breast cancer result in drug resistance or toxicity (3). ER is expressed in about 60% of all breast cancers and generally has a better prognosis and is often responsive to antiestrogen therapy; however, ER-independent breast cancers are more aggressive and unresponsive to antiestrogens (4).

Anticarcinogenic agents exert their cancer chemopreventive effects by modulating various biochemical and molecular pathways leading to the apoptotic cells death of cancer cells. Thus, induction of apoptosis in cancer cells has become an indicator of the cancer treatment response and reduction of mortality in cancer patients (5, 6). The treatment of cancer patients with chemotherapy often produces unsatisfactory and adverse toxic effects. Even the most commonly used drugs produce meaningful responses in <50% patients. Thus, too many patients are needlessly exposed to highly toxic drugs and suffer the side effects without much beneficial effects. Therefore, the search for nontoxic and more effective anticancer drugs or chemopreventive agents is required. Prevention of cancer via consumption of dietary supplements/nutraceuticals and beverages is now developing a considerable interest among common people. Green tea is consumed as a popular beverage worldwide (7). The anticarcinogenic effect of green tea polyphenols have been studied in several in vitro cell culture and in vivo animal models of various cancers like colorectal, ovarian, pancreatic, gastric, esophageal, prostate, breast, and skin cancers (7-10). The (−)-epigallocatechin-3-gallate (EGCG) is the major and most effective anticarcinogenic constituent found in green tea (7–10); however, its mechanism of action and cellular targets are poorly understood in breast cancers, and more...
specifically in ER-negative breast cancers. Chen et al. (11) have shown that EGCG induced growth inhibitory effects on cancerous cells but not on their normal counterparts. Kavanagh et al. (12) have shown that green tea extract decreases dimethylbenz(a)anthracene induced mammary tumor burden in Sprague-Dawley rats. We recently showed that the treatment of EGCG imparts a growth inhibitory effect on ER-positive human breast cancer MCF-7 cells via inhibition of telomerase activity (10). Aware of the fact that 40% of human breast cancers are ER negative and therefore unresponsive to antihormone therapy, we elucidated the anticarcinogenic effect of EGCG by using the MDA-MB-468 human breast cancer cell line as an in vitro model for ER-negative breast cancers.

The tumor suppressor protein p53 is considered to be a major player in the apoptotic response to genotoxins. Therefore, we were interested in elucidating the role of EGCG on p53-dependent pathway for the induction of apoptosis. P53 activation contributes to suppression of malignant transformation, and apoptosis has been characterized as a fundamental cellular activity to protect against neoplastic development in the organism by eliminating genetically damaged cells or those cells that have been improperly induced to divide by a mitotic stimulus (13, 14). The proteins of Bcl-2 family constitute proapoptotic and antiapoptotic regulators of apoptosis and their functions have been associated with the activation of tumor suppressor protein p53. The Bcl-2 protein binds to proapoptotic protein Bax and form heterodimers, and the molar ratio of Bax to Bcl-2 determines whether apoptosis is induced or inhibited in the target tissues (15). Bax is one of the primary targets of p53 and controls cell death through its participation in disruption of mitochondria with the subsequent release of cytochrome c in cytosols (16). Cytochrome c can interacts with apoptotic protease-activating factor-1 (Apaf-1) and leads to the activation of caspase-3 and poly(ADP-ribose) polymerase (PARP) which are the key mediators of apoptosis (17–20). Here, we report that EGCG induces apoptosis in ER-negative MDA-MB-468 breast cancer cells which is associated with enhanced expression of p53, Bax, and activation of caspase-3 and PARP cleavage.

Materials and Methods

Chemicals and Antibodies

Purified EGCG (>98%) was procured from Tokyo Food Techno Co. Ltd, Shizuoka, Japan. Annexin V–conjugated Alexa Fluor 488 Apoptosis Detection Kit was purchased from Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies for, phospho-p53 (p53 at Ser 15) and Bcl-2 were procured from Cell Signaling Technology, Inc. (Beverley, MA). Antibodies for p53, cytochrome c, Apaf-1, and ß-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for Bax and cleaved caspase-3 (specific for 19 and 17 kDa) were obtained from Cell Signaling Technology and PARP was obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). DMEM, penicillin, streptomycin, fetal bovine serum, and trypsin/EDTA were purchased from CellGro (Herndon, VA), and acrylamide and the protein assay kit were obtained from Bio-Rad (Hercules, CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals employed in this study were of analytic grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture Conditions

Human breast adenocarcinoma MDA-MB-468 cells were purchased from American Tissue Culture Collection (Manassas, VA). Cells were cultured in monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 µg/mL penicillin, and 100 µg/mL streptomycin, both from Invitrogen (Carlsbad, CA) and maintained at 37°C in a humidified atmosphere of 5% CO2.

 Colony Formation Assay

The antiproliferative effect of EGCG on MDA-MB-468 cells was assessed by colony formation assay, as described previously (10). Briefly, ~400 cells were plated into each well of the 6-well plates in triplicates for 24 hours. Thereafter, cell were treated with EGCG (1- 80 µg/mL). The cells were kept in incubator at 37°C for 14 days. It was expected that during this period each individual surviving cell would proliferate and form a colony. On day 15, the colonies were washed with PBS, fixed with 70% ethanol, and stained with 0.02% aqueous trypan blue solution. The colonies that had ≥50 cells per colony were counted. Data was represented as number of colonies formed with and without treatment of EGCG and converted to percentage with respect to non-EGCG treated control group. The colony-forming potential of MDA-MB-468 cells after EGCG treatment was expressed in terms of percent of control.

Cell Growth/Cell Viability Assay

The MTT assay was done to determine the cell growth and viability of MDA-MB-468 cells under the influence of EGCG as described previously (10, 21). Approximately 5,000 MDA-MB-468 cells were plated in each well of 96 well plates and kept in the incubator at 37°C. After overnight incubation, the cells were treated without or with EGCG (5-80 µg/mL) for 24, 48, and 72 hours. At the stipulated time following the treatment of EGCG, medium was aspirated and MTT (50 µL of 5 mg/mL) was added in each well and incubate at 37°C for 2 hours. The plates were spun, and purple colored precipitates of formazan were dissolved in 150 µL of dimethyl sulfoxide. The color absorbance was recorded at 540 nm on a BioRad 3350 microplate reader, taking 650 nm as a reference. The reduction in viability of MDA-MB-468 cells in EGCG-treated well was expressed as the percentage compared with non-EGCG treated control cells.

Detection of Apoptosis by DNA Ladder Assay

EGCG-induced apoptosis in MDA-MB-468 cells was determined in the form of fragmented DNA following the method of Laird et al. (22). Briefly, after treating MDA-MB-468 cells without or with EGCG at desired
concentrations and time intervals, cells were harvested, washed with PBS (pH 7.4) and incubated with lysis buffer [10 mmol/L Tris-HCl (pH 8.5), 5 mmol/L EDTA, 0.2% SDS, 0.2 mol/L NaCl, 0.1 mg/mL protease K] at 37°C for 2 to 3 hours. DNA was extracted by mixing an equal volume of isopropanol to the lysate. The lysate was then centrifuged at 3,000 rpm for 5 minutes to pellet down the DNA. The pelleted DNA was air-dried and resuspended in 500 μL of 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 7.5) for complete dissolution overnight at 55°C. One microgram of DNA was resolved over 0.8% agarose gel following gel electrophoresis. The gel was then stained with 0.5 μg/mL of ethidium bromide in 1× Tris-borate-EDTA buffer. The bands were visualized under a UV transilluminator followed by polaroid photography.

Detection of Apoptotic Cells by Fluorescence Staining

Cells were treated with various concentrations of EGCG for 24, 48, and 72 hours. After EGCG treatment, cells were washed with PBS and then fixed in 0.1% ice-cold paraformaldehyde for 10 minutes. The cells were then washed twice with PBS and stained with Hoechst 33342 (50 μg/mL) for 1 minute in the dark. Morphologic changes in cells were observed under fluorescence microscope by two pathologists in a blinded manner, and the percentage of apoptotic cells was scored counting at least 200 cells per treatment group. The experiment was repeated thrice, and the average percentage of apoptotic cells was determined for each treatment of EGCG and expressed as mean ± SD.

Measurement of Apoptotic Cells by Flow Cytometry

Finally, the quantitative analysis of apoptotic cell death caused by EGCG treatment was done by using the Annexin V-Alexa Fluor 488 Apoptosis Detection Kit following the manufacturer’s protocol. Briefly, MDA-MB-468 cells were treated without and with EGCG (20-80 μmol/L) for 24, 48, and 72 hours. Cells were harvested, washed with cold PBS and subjected to Annexin V-Alexa Fluor 488 (Alexa488) and propidium iodide staining in binding buffer at room temperature for 10 minutes in the dark. Stained cells were analyzed by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA) using CellQuest 3.3 Software. In the apoptotic assay kit, recombinant Annexin V conjugated to Alexa Fluor 488 fluorescent dye, which increased its sensitivity. The apoptotic cells stained with Alexa488 showed green fluorescence and present in the lower right (LR) quadrant of the histogram and the cells stained with both Alexa488 and propidium iodide showed red and green fluorescence, and present in the upper right (UR) quadrant of the fluorescence-activated cell sorting histogram.

Cell Lysates, Immunoprecipitation, and Western Blotting

Following treatment of MDA-MB-468 cells with EGCG at desired concentrations and time intervals, the cells were harvested, washed twice with cold PBS [10 mmol/L (pH 7.4)] and lysed with ice-cold lysis buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1.5 mmol/L MgCl2, 1.0 mmol/L EDTA, 1.0 mmol/L DTT, 1.0 mmol/L phenylmethylsulfonyl fluoride, 0.1% IGEPAL CA-630, 2.0 μg/mL leupeptin and 2.0 μg/mL aprotinin]. The resulting homogenates were centrifuged at 14,000 × g for 1 minute at 4°C. The pellets were incubated on ice for 30 minutes with 25 μL of nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1.5 mmol/L MgCl2, 1.0 mmol/L EDTA] for 20 minutes. The supernatant was separated and stored at −80°C for Western blotting. The lysates were also immunoprecipitated using p53 antibodies and protein G plus/protein A-agarose. The beads were washed thrice with PBS, and were examined for phosphorylated p53 protein specific to Ser 15. Nuclear fractions were prepared for the analyses of caspase-3 and PARP (23). Briefly, the cells were incubated on ice for 15 minutes with 0.4 mL of ice-cold lysis buffer in a microfuge tube. Then, 12.5 μL of 10% IGEPAL CA-630 was added and mixed. The suspension was centrifuged at 14,000 × g for 1 minute at 4°C. The pellets were incubated on ice for 30 minutes with 25 μL of nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1.5 mmol/L MgCl2, 1.0 mmol/L EDTA] for 20 minutes. The supernatant was separated and stored at −80°C for Western blotting. The lysates were then washed with PBS thereafter incubated with blocking buffer (5% nonfat dry milk in 1% Tween 20 in 20 mmol/L TBS (pH 7.5)) for incubating for 1 hour at room temperature followed by incubation with the appropriate primary antibody in blocking buffer for overnight at 4°C. The blot was then washed with PBS thereafter incubated with appropriate secondary antibody conjugated with horseradish peroxidase at room temperature for 2 hours. Protein bands were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Inc., Piscataway, NJ) and done autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, NY). To verify equal protein loading and transfer of proteins from gel to membrane, the blots were stripped and reprobed for β-actin using an anti-actin rabbit polyclonal antibody and thereafter followed same protocol as detailed above. The relative intensity of each band from different treatment groups was determined using computerized software program.

Statistical Analysis

Statistical analysis was done using Student’s t test to determine the statistical significance of difference in the absolute values of the clonogenic potential, cell viability and apoptotic cell death between the EGCG treated and non-EGCG treated groups. The chemopreventive effect of EGCG was considered significant at P < 0.05 where not mentioned.

Results

EGCG Treatment Inhibits Cellular Proliferation and Cell Viability of MDA-MB-468 Cells

To assess the antiproliferative or anticarcinogenic effect of EGCG in MDA-MB-468 cells, dose-dependent effect was determined by anchorage-dependent colony-forming assay and MTT assay. In colony-forming assay, the cells were
tested for their proliferation potential and formation of an individual colony. We observed that in vitro treatment of EGCG resulted in cytotoxic effects on the human breast ER-negative adenocarcinoma MDA-MB-468 cells (Fig. 1A), and markedly inhibited the colony forming potential at 10 to 80 µg/mL of EGCG treatment. There was a drastic decrease in the ability of the MDA-MB-468 cells to form colonies with increasing doses of EGCG (≥20-80 µg/mL). Doses of 60 and 80 µg/mL of EGCG completely inhibited the proliferation potential of cells, as colonies were not detectable by the end of the experimental time of 14 days. The effect of EGCG on the cell viability of MDA-MB-468 cells was further determined and confirmed by the MTT assay. MDA-MB-468 cells were treated with varying doses of EGCG (5-80 µg/mL) for 24, 48, and 72 hours (Fig. 1B). Treatment of EGCG with 20 µg/mL had significantly reduced the cell viability (28-39% inhibition, \( P < 0.05 \)) of MDA-MB-468 cells from 24 hours onwards up to 72 hours, whereas 45% to 78% reduction (\( P < 0.01-0.001 \)) in cell viability was observed at the concentrations of 40 to 80 µg/mL of EGCG at 24, 48, and 72 hours of treatment. These observations indicated the antiproliferating and anticarcinogenic potential of EGCG against ER-negative human breast carcinoma cells. In this experiment, we found that the doses of 20 µg/mL and onward of EGCG resulted in significant inhibition of cell viability; therefore, in further mechanistic studies, we used 20, 40, and 60 µg/mL doses of EGCG.

**EGCG Treatment Induces Apoptosis in MDA-MB-468 Cells**

We extended our study to examine whether breast cancer cells are going under apoptosis after EGCG treatment. Induction of apoptosis in MDA-MB-468 cells caused by EGCG treatment was assessed by using DNA ladder assay. Our data indicated that treatment of EGCG (20-60 µg/mL) to MDA-MB-468 cells for 48 hours markedly damaged or fragmented DNA molecules in cancer cells as evident by DNA ladder formation assay (Fig. 2A). Similar results were also obtained after 24 and 72 hours of EGCG treatment although there was more intense DNA damage after 72 hours. In further experiments, the EGCG-induced apoptosis in MDA-MB-468 cells was morphologically identified using fluorescence staining with Hoechst 33342, as shown in Fig. 2B–I. Cells were treated with EGCG (Fig. 2C, D, and E) and without EGCG (Fig. 2B) for 48 hours, and for 72 hours with EGCG (Fig. 2G, H, and I) and without EGCG (Fig. 2F). Fluorescence microscopy data indicated that EGCG treatment dose- and time-dependently induced apoptosis in breast cancer cells. In these observations, fragmented nuclei were observed in EGCG treated cells, as evident in Fig. 2C, D, E and G, H, I. Apoptotic cells having deformed nuclei were counted in a blinded manner. The number of apoptotic cells was found 18% to 30% after 48 hours of EGCG treatment (20-60 µg/mL), whereas 20% to 54% apoptotic cells were observed after 72 hours of EGCG (20-60 µg/mL) treatment, as summarized in Fig. 2J. We were further interested to quantify and verify the number of apoptotic cells induced by EGCG treatment to MDA-MB-468 cells. For this purpose we used flow cytometry, and the

Figure 1. EGCG treatment inhibits the proliferation potential and cell viability of ER-negative human breast cancer MDA-MB-468 cells in a dose- and time-dependent manner. A, cellular proliferation was assayed by the clonogenic assay as described in Materials and Methods. A survival percentage of 100 correspond to the number of colonies obtained with control (non-EGCG treated) plates. Treatment of MDA-MB-468 cells with ≥40 µg/mL of EGCG almost completely inhibited colony formation capacity. The experiment was repeated thrice, and each time point indicates the effect of EGCG on colony forming potential of the cells in terms of mean of percent of control ± SD. ND, not detectable. B, dose- and time-dependent effect of EGCG on the cell viability of MDA-MB-468 cells. Viability of cells was determined by the MTT assay as described in Materials and Methods. Significant inhibition in cell viability was observed with the treatment of 20 to 80 µg/mL concentrations of EGCG at 24, 48, and 72h time points. Columns, % control (non-EGCG) as a mean of eight replicates; bars, ± SD. *, \( P < 0.05 \) versus control (non-EGCG) group; **, \( P < 0.01 \) versus control (non-EGCG) group; ***, \( P < 0.001 \) versus control (non-EGCG) group.

Annexin V–conjugated Alexa Fluor 488 (Alexa488) and propidium iodide staining were used to analyze the percentage of apoptotic cells induced by EGCG treatment under various concentrations and time intervals. The number of apoptotic cells was counted as late apoptotic cells shown in the UR quadrant and early apoptotic cells as shown in the LR quadrant of the histograms, as described earlier (10, 24). We found that treatment of EGCG at the doses of 20 to 80 µg/mL for 24 hours (Fig. 3A), increased the number of early apoptotic cells (LR), respectively, from 12.4% to 31.0% in a dose-dependent manner compared with that of 2.6% in non-EGCG treated control cells, whereas the number of late apoptotic cells
had increased from 5.3% to 14.0% compared with that of 3.1% in non-EGCG treated cells. The total percentage of apoptotic cells (UR + LR) increased from 5.7% in non-EGCG treated MDA-MB-468 cells to 41.1% in 80 μg/mL of EGCG treatment for 24 hours. The induction of apoptosis was higher when cells were treated with EGCG for 48 and 72 hours. The number of early apoptotic cells had increased from 2.4% in non-EGCG treated cells to 14.5% to 18.2% by 20 to 80 μg/mL of EGCG treatment for 48 hours (Fig. 3B). Thus, total percentage of apoptotic cells (UR + LR) increased from 7.7% in non-EGCG treated cells to 46.8% following the treatment of MDA-MB-468 cells with EGCG in the doses of 20 to 80 μg/mL for 48 hours. Similarly, the treatment of EGCG for 72 hours to MDA-MB-468 cells further increased the apoptosis of breast cancer cells. The treatment of EGCG dose- and time-dependently increased the apoptosis after 72 hours of its treatment compared with 24 and 48 hours of treatment, as shown in Fig. 3C. Thus, the total number of apoptotic cells (UR + LR) increased from 8.0% in non-EGCG treated cells to 19.2% to 66.2% following the treatment with EGCG (20-80 μg/mL) for 72 hours. The significant induction of apoptosis after EGCG treatment indicated its anticancer effect against ER-negative human breast carcinoma MDA-MB-468 cells.

EGCG Treatment Increases p53 Expression and Its Phosphorylation in MDA-MB-468 Cells

The p53 tumor suppressor gene is by far the most commonly mutated gene in human cancers (25); however, wild-type p53 provides a protective effect against neoplastic changes and tumor growth (26). Treatment of MDA-MB-468 cells with EGCG resulted in dose-dependent increase in the expression of p53 protein (Fig. 4A). Because the protective effect of p53 against suppression of transformation is dependent on the phosphorylation of p53 at Ser 15 residue (27), we determined whether p53 is phosphorylated at Ser 15 in MDA-MB-468 cells after treatment with EGCG. For this purpose, we used a phospho-specific antibody against p53 at Ser 15. Western blot analysis revealed that the level of phosphorylation of p53 was increased by 2.0- to 2.5-fold after 48 hours of EGCG (20-60 μg/mL) treatment to MDA-MB-468 cells. Similar results were also obtained when EGCG was treated for 72 hours (Fig. 4B).
EGCG Treatment Decreases the Expression of Anti-apoptotic Protein Bcl-2 but Increases the Expression of Proapoptotic Protein Bax in MDA-MB-468 Cells

The homodimerization and heterodimerization of proteins of Bcl-2 family is important for transduction and integration of apoptotic signals and control of the permeability of mitochondria and endoplasmic reticulum membranes. The antiapoptotic protein Bcl-2 has been associated with the inhibition of apoptosis whereas increased expression of proapoptotic protein Bax has been often associated with the increased apoptosis in target cells (15, 28). We therefore reasoned if p53 was involved in EGCG-induced apoptosis in MDA-MB-468 cells, there would be decreased expression of Bcl-2 and increased expression of Bax protein in EGCG treated MDA-MB-468 cells. As shown in Fig. 4, treatment of EGCG resulted in dose-dependent reduction of Bcl-2 expression (Fig. 4C) and increased expression of Bax protein in EGCG treated MDA-MB-468 cells. In most of the cases the ratio of Bax/Bcl-2 proteins is the determining factor to transmit the apoptotic signal, we therefore analyzed this ratio by comparing the relative intensity of their bands at similar doses of EGCG and at similar time points studied, and found that the ratio of Bax/Bcl-2 was dose-dependently increased both at 48 and 72 hours after EGCG treatment (Fig. 4E). This observation further supports the fact that induction of apoptosis in MDA-MB-468 cells by EGCG is mediated through reduction in antiapoptotic protein Bcl-2 and increased expression of proapoptotic protein Bax and their ratio.

EGCG Treatment Induces Cytochrome c Release from Mitochondria, Induction of Apaf-1 and Activation of Caspase-3 and PARP Cleavage in MDA-MB-468 Cells

In mitochondrial pathway, proapoptotic members of the Bcl-2 family, such as Bax, associate with the mitochondria and direct the release of cytochrome c. Cytochrome c binds to Apaf-1 in cytosol and leading to the activation of pro-caspase-9. Following stimulation, initiator caspases cleave effector caspases, such as caspase-3. Cleaved caspase-3 is the executioner of apoptosis which cleave a broad spectrum of cellular targets, including PARP, thus subsequently leads to apoptosis (17, 18). For these reasons, we determined the effect of EGCG on the expression of these proteins. As shown in Fig. 5, Western blot analysis indicated that treatment of MDA-MB-468 cells with EGCG resulted in dose-dependent increase in cytochrome c release from the mitochondria.

Figure 3. Treatment of EGCG dose-dependently (20-80 μg/mL) and time-dependently (24-72h) induced apoptosis in human breast cancer MDA-MB-468 cells. Annexin V-Alexa Fluor 488 (Alexa488) Apoptosis Vybrant Assay kit was used to analyze the number of apoptotic cells by flow cytometry. Cells were incubated with Alexa488 and propidium iodide in binding buffer at room temperature, and followed manufacturer’s protocol. Stained cells were analyzed by flow cytometry. A, effect of EGCG on apoptosis in MDA-MB-468 cells. Cells were treated for 24h with or without EGCG. B, effect of EGCG on apoptosis in MDA-MB-468 cells. Cells were treated for 48h with or without EGCG. C, effect of EGCG on apoptosis in MDA-MB-468 cells. Cells were treated for 72h with or without EGCG. Treatment of EGCG dose- and time-dependently increased the percentage of apoptotic cells in MDA-MB-468 cells. LR quadrant indicates the percentage of early apoptotic cells (Alexa488-stained cells), and UR quadrant indicates the percentage of late apoptotic cells (Alexa488 + propidium iodide-stained cells).
mitochondria (Fig. 5A), induction of Apaf-1 (Fig. 5B), activation of caspase-3 (Fig. 5C) and PARP (Fig. 5D) proteins at 48 and 72 hours after EGCG treatment. Cleaved caspase-3 (19 and 17 kDa) is crucial for the apoptotic signal. Because the antibodies specific to caspase-3 only recognizes cleaved products of caspase-3 (17 and 19 kDa), the original basal level of caspase-3 in non-EGCG treated cells was not detected in the blot (Fig. 5C).

**Discussion**

Breast cancer is one of the most common cancers among women in both developed and underdeveloped countries. In contrast to ER-positive breast cancer, the ER-negative breast cancers lack the hormone-dependent cell proliferation pathway and possess an alternative regulatory pathway for the “acquired growth stimulation autonomy” (29), proving conventional antihormone therapy futile. Worldwide interest in green tea polyphenols as a cancer chemopreventive agent has been increasing because it is non toxic and is effective in a wide range of organs (7–10). Epidemiologic studies have shown that green tea consumption is linked to a decrease in the rate of development and recurrence of breast cancer (30). Treatment of EGCG (50 mg per kg per day, 14 days) reduced the growth of ER-positive human breast cancer MCF-7 cells implanted breast tumors in athymic nude mice by 40% (31). EGCG treatment inhibits cellular proliferation in estrogen receptor positive human breast cancer MCF-7 cells through telomerase inactivation (10). Komori et al. (32) have suggested that anticarcinogenic activity of green tea polyphenols is due to inhibition of interaction of estrogen with its receptors. Mechanism of EGCG inhibition of breast cancer proliferation and ER-dependent tumor growth is not likely to be via ER antagonism as shown by absence of ER-mediated responses in vivo (33). Our results indicate that EGCG is also effective in its anticarcinogenic potential on the ER-negative human breast cancer cells in vitro as evidenced by the inhibition of the colony forming potential and cell viability (Fig. 1). Mechanism of EGCG mediated inhibition of human breast cancer cell proliferation and cell viability had to be highlighted to unravel the ER-independent

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Figure 4. Treatment of MDA-MB-468 cells with EGCG increases the expression of tumor suppressor protein p53 (A), and phosphorylation of p53 at Ser 15 residue (B). Treatment of EGCG to MDA-MB-468 cells also decreases the expression of anti-apoptotic protein Bcl-2 (C), and increases the expression of proapoptotic protein Bax (D). The relative intensities of bands in each blot were determined. The resultant ratio of Bax and Bcl-2 protein expression is shown as mean ± SD from three independent experiments (E). Cells were treated with different concentrations of EGCG (20, 40, and 60 μg/mL) for 48 and 72 as indicated. The expressions of different proteins were analyzed by Western blotting as detailed in Materials and Methods. A representative blot is shown from three independent experiments with identical results.
EGCG is shown. Relative intensity of bands in independent experiments with identical results. Treatment pattern of caspase-3 (19 and 17 kDa). A representative blot is shown from three antibody for caspase-3 specifically recognizes the cleaved products of 468 human breast cancer cells, as analyzed by Western blot analysis. (Apaf-1, B) was determined. 

C

Figure 5. Treatment of EGCG increases the release of cytochrome c (A), and increases the expression of apoptotic protease-activating factor-1 (Apaf-1, B), cleaved caspase = 3 (C) and cleaved PARP (D) in MDA-MB-468 human breast cancer cells, as analyzed by Western blot analysis. Antibody for caspase-3 specifically recognizes the cleaved products of caspase-3 (19 and 17 kDa). A representative blot is shown from three independent experiments with identical results. Treatment pattern of EGCG is shown. Relative intensity of bands in A and B was determined. ND, not detectable.

mechanism of its anticancer effect. We observed that inhibition of cell viability by EGCG was associated with the induction of apoptosis in ER-negative human breast cancer MDA-MB-468 cells, which was determined and confirmed by DNA ladder assay, fluorescence staining and flow cytometry (Figs. 2 and 3). Apoptosis has been characterized as a fundamental cellular activity, and plays a crucial role as a protective mechanism against neoplastic development in the organisms by eliminating genetically damaged cells or those cells that have been improperly induced to divide by a mitotic stimulus (13, 14). Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Therefore, green tea seems to be a potent anticarcinogenic agent for breast cancer patients.

Because the breast cancer represents the most commonly diagnosed invasive malignancy in women and because the treatment options including surgery have not been able to deal with the growing incidence of breast malignancy, it is required to develop mechanism-based novel agents for its prevention. Therefore, our aim was to identify the mechanism of EGCG-induced apoptosis in ER-negative MDA-MB-468 cells. P53 tumor suppressor gene and its product are key components of the DNA damage sensors that can induce the apoptotic effector cascade. In >50% of human cancers, the p53 protein is functionally inactivated resulting in resistance to induction of apoptosis (34). In this study, we found that EGCG increases the expression of p53 protein and its phosphorylation at Ser 15 in MDA-MB-468 cells thereby paving the way for the cells to be easily susceptible to apoptosis leading to cellular mortality (Fig. 4A and B). Phosphorylation of p53 has been associated with cellular damage occurring during anticancer therapy. Moreover, p53 phosphorylation at Ser 15 increases the half-life of p53 protein and thus increases the accumulation and functional activation of p53 in response to DNA damage, thereby stabilizing it (35, 36). This information provides evidence that increase in p53 protein expression after EGCG treatment may be due to increased stability. P53 has been shown to exert its tumor suppressing activity by regulating the process of apoptosis (37, 38), and this fact is supported by the fact that lack of p53 expression or function is associated with an increased risk of tumor formation (39, 40). Moreover, inhibition of apoptosis is considered as one of the possible mechanisms of tumor development (41, 42), therefore, our data indicated that the induction of apoptosis by EGCG may be one of the chemopreventive mechanisms for the ER-negative breast cancer cells.

Tumor suppressor protein p53 induced apoptosis through several pathways and one of these involves the proapoptotic and antiapoptotic proteins of Bcl-2 family. The Bcl-2 family elicits opposing effects on mitochondria. Bax protein is a p53 target and a proapoptotic member of the Bcl-2 family (43, 44) and can promote the release of cytochrome c from mitochondria, which in turn activates caspase-3, one of the key executioners of p53-mediated apoptosis, and PARP (18–20). The antiapoptotic proteins, such as Bcl-2, which are transcriptionally suppressed by p53 help to preserve the integrity of the mitochondria (45). This suppresses the release of cytochrome c that activates the effectors of apoptosis (46). We observed that EGCG increases the expression of Bax and reduces the expression of Bcl-2 in MDA-MB-468 cells (Fig. 4). The change in the ratio of Bax/Bcl-2 is critical for the induction of apoptosis, and this ratio decides whether or not cells will go for apoptosis (19, 47, 48). Alteration in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into cytosol. Cytosolic cytochrome c binds to Apaf-1 and leads to the activation of caspase-3 and PARP (17, 18). Treatment of EGCG to ER-negative human breast cancer MDA-MB-468 cells results in an increase in Bax protein while decrease in the expression of Bcl-2, and thus increased ratio of Bax/Bcl-2 (Fig. 4E) may be responsible for the release of cytochrome c from mitochondria (Fig. 5A), increased expression of Apaf-1 (Fig. 5B), and finally increased expression of cleaved caspase-3 and PARP (Fig. 5C and D). These results provide evidence for the involvement of p53 and its phosphorylation at Ser 15 in EGCG-induced apoptosis in ER-negative human breast cancer cells.
In conclusion, the data from our study indicate that EGCG induced apoptosis in ER-negative human breast cancer MDA-MB-468 cells is mediated through the tumor suppressor protein p53-dependent pathway which involves the proteins of Bcl-2 family and activation of caspase-3 and PARP. The results of our study also provide evidence that EGCG is an effective chemopreventive agent for ER-negative human breast cancer cells, and thus providing the molecular basis for further studies in in vivo system and the development of EGCG as a novel and safe chemopreventive agent for breast cancer prevention. It is important to mention that in the present study, treatment of EGCG is killing the cancerous cells, and this should be considered as a high risk situation although the cancerous cells are in direct contact with the anticarcinogenic agent. We believe that in chemopreventive approach continuous lesser dose of EGCG should be sufficient to prevent the incidence of breast cancer risk in healthy human population. Based on some epidemiologic and experimental studies, it has been suggested that consumption of six to seven cups of green tea per day should provide the chemopreventive effects against the cancer risk. However, studies are warranted to examine the chemopreventive effect of green tea against breast cancer in high risk human population.

References


Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation

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