Abstract. Recent investigations have demonstrated that polyphenolic catechins inhibit cancer cell proliferation and tumor growth. However, how the major active component of tea catechins, epigallocatechin-3 gallate (EGCG), mediates anticancerous effects has not been extensively examined. We have investigated the cell growth inhibitory effects of EGCG on cell growth of the human breast cancer cell line MCF-7, and the mechanism of its action with emphasis on the regulation of tumor cell survival. A significant EGCG dose-dependent growth inhibition was observed coordinated with EGCG-induced apoptosis. Analysis of survivin expression after addition of EGCG showed that both survivin mRNA and protein were decreased. The survivin-promoter luciferase activity in EGCG-treated cells was significantly inhibited by 91±2.0% (P<0.001), compared with the control. Interestingly, EGCG strongly inhibited the basal activation of phospho-AKT and AKT kinase activity as early as 30 min after treatment. Furthermore, inhibition of AKT kinase activity by EGCG preceded the suppression of survivin (1 h post treatment), followed by increased caspase-9 activity (6 h post treatment). A dominant negative AKT or the phosphatidylinositol 3-kinase inhibitor, LY294002, also strongly inhibited survivin promoter activity, providing further evidence to support the hypothesis that the inhibitory effect of EGCG on survivin is mediated via the AKT pathway. Therefore, EGCG is a potent proapoptotic agent in MCF-7 breast cancer cells that targets survivin expression via suppression of the AKT pathway.

Introduction

Both epidemiological and laboratory studies have positively shown an association of tea consumption with lower risk for certain types of cancers (1-4). The cancer preventive and therapeutic potential of tea has received more attention in recent years largely due to the purification and studies of the effective anti-tumorigenic component, the tea polyphenols, known as catechins. There are four major catechins in green tea of which the most effective and predominant component is epigallocatechin-3 gallate (EGCG). Previous studies have shown that plasma concentration of EGCG reached a peak level between 1.5 and 2.5 h after drinking beverages containing 1.5-4.5 g dried green tea (DGT) (about 400-1500 mg EGCG) (5-8). Steady-state plasma levels of EGCG can be maintained with daily beverage drinking (7). Not only could EGCG inhibit tumor progression at different stages including cancer initiation, promotion, and progression but it could suppress different types of carcinomas such as skin, lung, oral cavity, esophagus, stomach, small intestine, colon, and pancreas (9-19). Breast cancer will affect 175,000 women and 1300 men in the US each year as estimated by the American Cancer Society. Although the epidemiologic studies showed a possible association of tea consumption in Asian Americans and a lower incidence of breast cancer (20), mixed laboratory results were reported (21). However, direct or indirect inhibitory effects of EGCG on mammary tumor cells have been reported though the mechanisms are still not clear (22-24).

The proposed advantage of EGCG as a chemopreventive agent relies on its selective elimination of transformed or neoplastic cells, while sparing or in some cases protecting normal cells (19,25,26). This selectivity may be rooted in EGCG's ability to target abnormally expressed cell signaling or anti-apoptotic proteins in tumor cells but not in normal cells.

Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, is one such target that is widely expressed in fetal tissues and in human cancers, but is nearly absent or found at miniscule levels in normal adult tissues (27-33). Survivin contains a baculovirus inhibitor of apoptosis repeat (BIR) protein domain that inhibits apoptosis by either directly or indirectly interfering with the function of caspases (27). The tumor-specific expression of survivin, coupled with its
importance in inhibiting cell death makes it a useful diagnostic marker of cancer and a potential target for cancer treatment. In studies of primary breast carcinoma (34), especially ductal carcinoma (35), survivin is overexpressed and may serve as a prognostic indicator of worse outcome (36). Studies by Altieri's group have shown that a dominant negative mutant survivin (pAd-T34A) inhibited growth of established tumors and triggered tumor cell apoptosis (37,38) in vivo while wild-type survivin inhibited growth factor withdrawal- and ceramide-induced apoptosis. Therefore, survivin may be a valid target for EGCG in human breast cancers.

In this report we demonstrate that EGCG exerts its inhibitory activity in breast cancer via its ability to promote apoptosis through selectively inhibiting survival/anti-apoptotic pathways in breast cancer cell line, MCF-7, by direct or indirect suppression of survivin mRNA and protein.

Materials and methods

Cell culture. MCF-7 cells (N variant) (39) were maintained and grown in a 10% completed medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), BME amino acids, MEM amino acids, glutamine, penicillin/streptomycin, sodium pyruvate (Invitrogen, Carlsbad, CA) under mycoplasma-free conditions. MCF-7 cells were grown for 2 days in a 5% CS medium (DMEM phenol-red-free, with 5% dextran-coated charcoal stripped FBS) containing BME amino acids, MEM amino acids, glutamine, penicillin/streptomycin, sodium pyruvate.

Colony assay. Cells were dispersed into 6-well plates containing 500 cells/well. To each well of the 6-well plates, 2 ml of the 5% CS growth medium was added. Plates were incubated in a 5% CO2 incubator at 37°C. After 24 h of growth, the cells were treated with EGCG (LKT lab, St. Paul, MN) with increasing concentrations of 10-50 μg/ml for 48 h. Treatment-containing media were removed after 48 h and replaced with fresh 10% FBS complete medium, and then the cells were cultured for ~14 days. The cells were fixed with 5% glutaraldehyde for 10 min, washed with PBS once, and then stained with 5% crystal violet for 20 min. The numbers of colonies (≥50 cells) were counted with an Oxford Optronix COLCount (Oxford, UK).

Viability assay. MCF-7 cells were plated in 96-well plates at 1x10⁴ cells per well in 10% DMEM. The cells were allowed to adhere for 48 h and were treated appropriately. Forty-eight hours later the media was removed, and the cells were stained with 0.1 ml of 0.5% crystal violet solution for 10 min, washed with PBS to remove excess crystal violet and lysed in 0.1 ml of 0.1% SDS solution. Absorbance at 540 nM was measured using a 96-well microplate reader. Percent viability was determined based on normalization to untreated control cells (set at 100%). Decreased viability represents a decrease in percentage of staining as compared with 100% control values.

Apoptotic assay. The MCF-7 cells were grown to 80% confluence in a 4-well chamber slide (Nalge Nunc, Naperville, IL) and treated with or without 50 μg/ml EGCG for 48 h. Cells were washed twice with PBS and then stained by an Annexin-V apoptosis detection kit (Molecular Probes, Eugene, OR) as instructed by the manufacturer's protocol. FITC-stained apoptotic cells were examined using fluorescence microscopy (Leica, Wetzlar, Germany).

RT-PCR. Total RNA was isolated from MCF-7 cells using TRIzol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA), and quantified by UV absorbance. The reverse transcription and PCR were performed using the superscript system for first-strand cDNA synthesis and PCR Supermix (Invitrogen), respectively. The reverse transcription of RNA was performed in a final volume of 20 μl containing 1X PCR buffer, 2.5 mM MgCl₂, 0.5 mM dNTP mix, 10 mM DTT, 10 U of SuperScript II, 25 ng/μl oligo (dT), 1 μg of total RNA and DEPC-treated water. After incubation at 42°C for 50 min, the reaction was terminated by raising the temperature to 70°C for 15 min. For PCR, Supermix containing 200 nM of primers was added to 1 μl of the newly synthesized cDNA to bring the final volume to 25 μl. The reaction mixture was first heated at 94°C for 5 min and amplification was carried out in 35 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, followed by a final incubation at 72°C for 5 min, with a Perkin-Elmer 2400 Thermocycler (Perkin-Elmer, Norwalk, CT). The oligonucleotide primers specific for survivin and GAPDH (survivin: forward primer, 5'-TGGCCCGACGGTGCC-3'; reverse primer, 5'-CAGTCTTTGAGTGTAGATGGTGT-3'; GAPDH: forward primer, 5'-GAAGGATTAGTTGAGGAGAT-3'; reverse primer, 5'-GAAGATGATGATGGATG-3') were used (41,42). PCR products were fractionated on 2% agarose gel and visualized with ethidium bromide staining.

Western blot analysis and kinase assay. MCF-7 cells were harvested in sonicating buffer (62.5 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 2.5 μg/ml aprotinin and 1 mM Na orthovanadate) sonicated for 30 sec. Following centrifugation at 1000 x g for 20 min, 50 μg of protein was resuspended in sample buffer [62.5 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue], boiled for 5 min. Denatured proteins were separated by electrophoresis on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad Hercules, CA) in 20 mM Tris base, 150 mM glycine, and 20% v/v...
Figure 1. A, EGCG reduces survival of MCF-7 cells. A, colony survival inhibition of MCF-7 cells. Cells were treated with vehicle (DMSO) or EGCG (10-50 mg/ml) for 2 days then cultured for 2 weeks. See details in Materials and methods. The data represent means ± SEM (n=9). B, viability inhibition of MCF-7 cells. Cells (1x10^4/well) were plated in 96-well plates and treated with DMSO or EGCG (10-100 mg/ml) for 2 days, and stained with 0.5% crystal violet. Cells were lysed with 1% SDS and absorption was obtained at 540 nm. The data represent means ± SEM (n=9). C, apoptosis induction in MCF-7 cells. MCF-7 cells (80% confluent) in a 4-well chamber slide were treated with EGCG (50 mg/ml) or not for 2 days. Cells were washed twice with PBS and then stained by an Annexin-V apoptosis detection kit (Molecular Probes) as instructed by the manufacturer’s protocol. FITC-stained apoptotic cells were demonstrated in fluorescence microscopy (x10).
methanol for 100 V/h. Non-specific binding was blocked by incubating the blot with PBS-Tween (0.05%)-5% low-fat dry milk solution for 1 h at room temperature. The membrane was subsequently incubated overnight at 4°C with rabbit survivin monoclonal antibodies, 1:1000 dilution (Pharmingen, San Diego, CA), phospho-AKT antibody (Cell Signaling Technology, Beverly, MA), caspase-9, (Oncogene, San Diego, CA) and β-actin (Sigma). Blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:10,000 dilution; Gaithersburg, MD) for 60 min at room temperature. Following washes with PBS-Tween solution, immunoreactive proteins were visualized with ECL reagent (Amersham, Arlington Heights, IL) and then exposed to Hyperfilm (Amersham).

AKT kinase activity assays were performed according to the manufacturer's instructions (Cell Signaling Technology). Briefly, cell lysates with 200 μg protein from EGCG-treated MCF-7 cells with treatment times ranging from 0.5 to 24 h or from untreated (0 h) cells. Cells were mixed with immobilized AKT antibody slurry overnight at 4°C. Immunoprecipitated protein was washed twice with ice-cold lysis and kinase buffer, then incubated with 1 μg GSK-3 fusion protein and 200 μM ATP for 30 min at 30°C. The reaction was terminated by adding 3X SDS sample buffer and boiling for 5 min. The sample (30 μl) was loaded on 12% SDS-PAGE gel. Specific band was identified by anti-phospho-3 α/β SK fusion protein antibodies, and visualized as described above.

Statistical analysis. Statistical differences in colony formation, and survivin promoter activity with or without EGCG were determined by a Student's t-test, and the data are expressed as means ± SEM.

Results

The effect of EGCG on cell colony formation in a dose-dependent manner is shown in Fig. 1A. When MCF-7 cells were treated with 10 or 30 μg/ml of EGCG, colony survival was reduced to 79 and 67% respectively as compared to untreated control plates (100%). At 50 μg/ml of EGCG the percent of colony survival was significantly reduced to 38%±8% (P<0.001). When treated with 100 μg/ml of EGCG, all the cells were unable to form visible colonies. The ability of EGCG to affect long-term colony formation may be in part through both suppression of and/or induction of cell death. The effects of EGCG on cellular viability of MCF-7 cells were detected with a crystal violet assay (Fig. 1B). As shown in Fig. 1B, a similar and significant dose-dependent inhibition of cell proliferation was observed with 24-h treatment with EGCG (P<0.001), reducing viability to 68% when compared with untreated control group.

Using Annexin-V apoptosis detection kits the untreated and treated MCF-7 cells were tested for apoptosis induced by EGCG. Fifty μg/ml EGCG was able to induce apoptosis in MCF-7 as detected in Annexin-V binding assay, indicating EGCG as a potent inducer of apoptosis in MCF-7 cells (Fig. 1C).

The mechanisms involved in the inhibition of growth and induction of apoptosis by EGCG have not been fully explored. To test whether EGCG-induced apoptosis of MCF-7 cells involves altered survivin expression, RT-PCR and Western blotting were used to detect endogenous production of survivin mRNA and survivin protein. As shown in Fig. 2A, a high level expression of survivin mRNA and survivin were observed in MCF-7 cells (Fig. 2A, control lane) which was minimally affected by 1 ng/ml TNF-α (Fig. 2A, lane 2). In contrast, 50 μg/ml EGCG treatment for 24 h (Fig. 2A, lane 3) strongly inhibited both survivin mRNA and protein expression as compared to control and TNF-α-treated groups. Using a reporter construct of the survivin promoter linked to luciferase we further demonstrated that treatment of MCF-7 cells with 50 μg/ml of EGCG for 18 h reduced promoter activity by 91±2.0% as compared to DMSO-treated controls (100%) (Fig. 2B) (P<0.001).

Survivin has been demonstrated to be up-regulated by the AKT signaling pathway (43). To test the hypothesis that the AKT signal transduction pathway is involved in the survivin promoter regulation, a DN-AKT was transfected into MCF-7 cells. As demonstrated in Fig. 3, a trans-activation survivin promoter in SP13-transfected MCF-7 cells was significantly inhibited by DN-AKT. Furthermore, LY294002 (30 μM), a specific inhibitor of the phosphatidylinositol 3-kinase (PI3K)/AKT signal transduction pathway, was able to significantly block the transactivation of survivin promoter (Fig. 3) (P<0.01).

Figure 2. A, suppression of expression of both protein and mRNA in MCF-7 cells. Cells were preincubated with 50 ng/ml egcg for 24 h. Cell lysates were obtained as described in Materials and methods. Control (Ctr) treatments were with vehicle (DMSO) or 1 ng/ml tumor necrosis factor alpha (TNF-α). One mg RNA or 50 mg protein was used for RT-PCR or Western blot analyses, respectively. B, prevention of transactivation of survivin promoter. Cells (in 24-well plates) were transfected with 200 ng of pGL3-survivin-luciferase plasmid (SP13) using Effectene transfection reagent according to the manufacturer's protocol (Qiagen) for 5 h, and treated with vehicle (DMSO) or 50 mg/ml of EGCG for 18 h. Luciferase activity was obtained using 30 ml of cell extract. The data were normalized per microgram protein. The data represent four replicates.
In order to investigate whether the AKT pathway is involved in survivin inhibition induced apoptosis by EGCG, phosphorylated AKT expression levels were determined by Western blot analysis with specific anti-phospho-AKT (Ser-473) antibodies (Fig. 4). Expression of survivin and levels of phosphorylated AKT in control and TNF-α treatment groups were similar. However, both survivin and phosphorylated AKT were obviously decreased in the EGCG-treated group.

The relationship between AKT and survivin and apoptosis in MCF-7 cells treated with EGCG was studied using Western blot assays as demonstrated in Fig. 5. AKT kinase activity was assessed using immunoprecipitates from cellular extracts obtained with total AKT antibody and GSK-3 as a substrate. Interestingly, AKT activity dramatically decreased in 30 min after treatment with EGCG and the effect lasted for 24 h. However, survivin was not immediately inhibited by EGCG until 3 h after the treatment. Activated caspase-9 accumulated at 6 h.

**Discussion**

In this study we demonstrated that EGCG effectively inhibited colony formation and induced apoptosis of MCF-7 breast cancer cells via down-regulation of survivin, a major member of the IAP gene family. EGCG, one of the most important polyphenols in green tea, is demonstrated to be a potent growth suppressor of certain cancer xenografts, as well as decreasing tumor vessel density in vivo and in vitro (22,23). The inhibition of cell growth and the induction of apoptosis by EGCG have been demonstrated in a number of cell lines (1,44). EGCG (2-5 μM) inhibited TPA- or EGF-induced transformation by inhibition of AP-1 transcriptional activity (24,37). In this experiment, treatment of MCF-7 cells with EGCG for 48 h resulted in a dose-dependent inhibition of colony formation (Fig. 1A and B). Doses as low as 50 μg/ml of EGCG significantly inhibited colony formation in these cells (P<0.01). As mentioned above, the inhibitory effect of EGCG was tumor-specific and was not observed in the normal counterparts of the tumor cell line (20). Our study suggests and supports the previous results that EGCG is a potent in vitro inhibitor of MCF-7 cell growth at 50 μg/ml.

The mechanism of EGCG effects on colony formation of cultured cancer cells has not been adequately explored except for a few published results that associated the inhibition of neoplastic proliferation by EGCG with evidence of blocking effects on Her2/Neu signaling (45), G1/S arrest by induction of p21 (CIP1/WAF1/SDI1), inhibition of cyclin D1-associated pRB kinase activity, or impairment of pRB phosphorylation (46). Details of cell cycle control by EGCG, however, are still not well understood. Early studies with EGCG in a breast cancer cell line suggested that it could induce apoptosis (47), and new results linked growth inhibition with the induction of apoptosis by EGCG (48-51), but the effective concentration is higher than that required for growth inhibition. Consistent with previous reports on breast cancer cells (44), accompanied with our observation demonstrating reduced colony formation, a dramatic increase in apoptosis was observed in EGCG-treated MCF-7 cells (50 μg/ml) (Fig. 1B and 1C). Molecular evidence of induction of apoptosis in MCF-7 cells by EGCG was provided at different levels of the apoptotic signaling network.
pathways in our study. First, a high basal level of survivin mRNA and protein (Fig. 2A, control lanes) were present in cultured MCF-7 cells that is consistent with previous reports that cultured breast cancer cells express high levels of survivin (34-36,52). The level of constitutive survivin mRNA and protein (Fig. 2A) in MCF-7 cells was minimally inhibited by TNF-α (Fig. 2A, lane 2), a potent apoptotic inducer in many cells, but significantly inhibited by EGCG (50 μg/ml) (Fig. 2A, lane 3), which may suggest the presence of a different pathway of apoptosis induced by EGCG. Interestingly, EGCG seems to exert its effect on the trans-inhibition of survivin promoter activity (Fig. 2B). Finally, an increase in activated caspase-3 levels was observed after 6-h treatment with EGCG (50 μg/ml) (Fig. 5, lane 5), consistent with apoptosis.

Akt is a serine (Ser)/threonine (Thr) protein kinase which resides within the cytosol in a catalytically inactive state in quiescent or serum-starved cells. After stimulation of cells with growth factors and cytokines, Akt is catalytically activated by phosphorylation at Thr308 and Ser473. Activated Akt in turn phosphorylates downstream target molecules such as BAD which promote induction of its anti-apoptosis effect (53-55). Most interestingly survivin was upregulated by the AKT signaling pathway (43). We confirmed that the pharmacological inhibitor of the PI3K/AKT pathway (LY294002) (56) or a dominant-negative Akt (K179M) strongly inhibited survivin expression and survivin promoter activity in MCF-7 cells (Fig. 5). Interestingly, EGCG also strongly inhibited phosphorylation of AKT and AKT kinase activity in MCF-7 cells (Figs. 4 and 5), suggesting that EGCG induces apoptosis in MCF-7 cells by inhibiting constitutive activation of AKT and subsequent survivin expression. Taken together, these results strongly suggest that decreased expression of PI3K/AKT is a necessary step for the negative regulation of survivin gene expression by EGCG.

Human caspase-9, a member of the protease family intimately associated with the initiation of apoptosis, is able to be phosphorylated and inhibited by Akt (60). We demonstrated the time course of caspase-9 activation after treatment of EGCG at 6 h, compared with control, preceded by the decrease in phosphorylated Akt (30 min), and decreased expression of survivin (3 h post-exposure). The time course of inhibition of AKT kinase activity, suppression of survivin, and activated caspase-9 in response to EGCG strongly suggests that the inhibitory effect of EGCG on breast cancer cell growth results from induction of apoptosis via an AKT-survivin-dependent mechanism.

Survivin is one of the specific classes of inhibitors of apoptosis proteins (IAPs), and plays a pivotal role in tumor proliferation and resistance to chemotheraphy or radiation therapy in breast cancers. In this study we demonstrated that inhibition of survivin either directly or indirectly by EGCG is a key step in the molecular mechanism involved in the antiproliferative and proapoptotic effects of green tea which may contribute to its potential use for breast cancer therapy and/or prevention.

References


