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Green Tea Catechins: Proposed Mechanisms of Action in Breast Cancer Focusing on the Interplay Between Survival and Apoptosis

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Abstract: Recent data have shown strong chemopreventive and possibly cancer chemotherapeutic effects of green tea polyphenols against cancer. Despite advances in breast cancer treatment, mortality from breast cancer is still high. Undoubtedly novel treatment strategies are needed for chemoprevention of high risk women and for the treatment of receptor negative breast cancer. Green tea catechins have been shown to inhibit proliferation of breast cancer cells and to block carcinogenesis. This review attempts a critical presentation of the mechanisms of action of green tea catechins in breast cancer. Several mechanisms of action of green tea catechins in breast cancer have been proposed including modulation of extracellular signalling, induction of apoptosis through redox regulation, or through modulation of epigenetic alterations. A number of molecular targets of green tea catechins have been suggested i.e molecular chaperones, telomerase, apoptotic cascade. Although the molecular links among the proposed mechanisms of action of green tea catechins are often missing, it must be emphasized that all the proposed mechanisms indicate that green tea catechins inhibit growth and /or promote apoptosis. It would be interesting if future experimental trials could take into account that green tea catechins are multi-target agents and attempt to link every novel proposed target with the other already proposed targets of green tea catechins.

Keywords: Green tea catechins, breast cancer, EGCG, carcinogenesis, apoptosis, mechanisms of action.

INTRODUCTION

Polyphenols, particularly flavonoids constitute the most interesting component of green tea leaves. Catechins (flavan-3-ols) are the main flavonoids present in green tea. The four major catechins are (-)-epigallocatechin-3-gallate (EGCG), that represents approximately 59% of the total of catechins; (-)-epigallocatechin (EGC) (approximately 19%); (_)-epicatechin-3-gallate (ECG) (approximately 13.6%); and (_)-epicatechin (EC) (approximately 6.4%) [1]. The potential cancer chemopreventive and therapeutic properties of teas and tea polyphenols have been in the focus of research efforts in the last two decades. Recent data have shown strong chemopreventive and possibly cancer chemotherapeutic effects of green tea polyphenols and EGCG against cancers of the skin (UV radiation and chemically induced), lung, breast, colon, liver, stomach, prostate [2-5]. It is estimated that 1,200,000 new breast cancer cases are diagnosed annually worldwide [6]. Despite advances in breast cancer treatment, mortality from breast cancer is still high. Undoubtedly novel treatment strategies are needed especially for chemoprevention of high risk women and for the treatment of receptor negative breast cancer. Green tea catechins have been shown to inhibit proliferation of breast cancer cells and to block carcinogenesis. Several mechanisms of action of green tea catechins in breast cancer have been proposed. A number of molecular targets of green tea catechins have been suggested. Different mechanisms and targets have been investigated in different trials. Although the molecular link between the different targets is not always obvious, critical review of the literature suggests that green tea catechins inhibit growth and pro-survival pathways and turn on pro-apoptotic pathways. This review aims to critically synthesize evidence on the mechanism of action of green tea catechins in breast cancer focusing on the interplay between survival and apoptosis.

LITERATURE SEARCH STRATEGY

Pubmed, Scopus,Google Scholar, Science Citation Index were searched with the search terms “EGCG”, “green tea catechins” “breast cancer”, “EGC”, “ECG”, “catechin hydrate”, “mechanisms of action”, “apoptosis”, “oxidative stress”, “redox modulation”, “cell cycle regulation”. The search covered the period from 1966 up to and including February 2013. Experimental *in vitro* and *in vivo* studies, that investigated the mechanism of action of green tea catechins on breast cancer met inclusion criteria. Exclusion criteria: (i) studies that investigated the potential chemopreventive or therapeutic effect of green tea catechins on breast cancer without further investigating the mechanisms of action and the putative molecular targets of green tea catechins; (ii) given that green tea catechins are multitarget agents, studies that investigated the effect of green tea catechins on a single molecular target were excluded (iii) studies that investigated the mechanisms of action of green tea catechin analogs (iv) studies that investigated the mechanism of action of green tea catechins when co-administered with other treatments (v) studies that investigated the effect of green tea catechins in other malignant tumors. Only full publications were considered.

The majority of the studies were *in vitro* studies with only one *in vivo* study being identified (Table **1**). The experimental model in the *in vivo* study was the C3(1) SV40 Tag mouse model that has been used extensively for the evaluation of the effects of interventional and preventive therapies as well as for the investigation of the molecular and genetic alterations occurring at various stages of disease progression.

MODULATION OF SIGNALLING PATHWAYS OF CELL GROWTH AND SURVIVAL

Modulation of Extracellular Signalling

Rathore *et al.* [7-9] have shown that green tea catechins at a non-cytotoxic, physiologically achievable concentration of 2.5 μg/mL suppressed the NNK- and B[a]P-carcinogen-induced cellular carcinogenesis in human breast epithelial MCF10A cells as measured by reduced dependence on growth factors, anchorage-independent growth, increased cell mobility, and acinar conformational disruption. Cumulative exposure of MCF10A cells to the NNK- and B[a]P-carcinogens results in the aquisition of cancer related properties. In that study, the authors investigated the effect of various concentrations of green tea catechins on

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Table 1. Main Mechanism of action of green tea catechins in breast cancer

Author	Experimental Model	Green Tea Catechin	Main Mechanism
Rathore *et al*. 2012, Rathore *et al*. 2013	MCF10A cell line	EC, ECG, EGC and EGCG	Modulation of extracellular signalling
Bigelow and Cardelli	MCF10A cell line	EGCG	inhibition of hepatocyte growth factor signaling
Tran *et al*.	MCF7 cell line	EGCG	Inhibition of molecular chaperones
Kaur *et al*.	C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma (TAg) mouse model	Green tea extract	Redox modulation and induction of apoptosis
Hsuuw *et al*	MCF7 cell line	EGCG	Low concentrations of EGCG induce apoptosis, while high concentrations induce necrotic cell death
Mittal *et al*.	MCF7 cell line	EGCG	Induction of apoptosis *via* epigenetic downregulation of telomerase
Meeran *et al*.	MCF7 and MDA-MB-231 cell line	EGCG	Induction of apoptosis *via* epigenetic downregulation of telomerase
Belretch *et al*	MCF7 cell line	EGCG	Induction of apoptosis *via* epigenetic downregulation of telomerase
Alshatwi *et al*.	MCF7 cell line	Catechin hydrate	Directly targeting apoptotic cascade
Roy *et al*.	MDA-MB-231 cell line	EGCG	Directly targeting apoptotic cascade
Vergote *et al*.	MCF-7 and MDA-MB-231 cell line	EGC	Directly targeting apoptotic cascade
Landis Piwowar *et al*.	MDA-MB-231 cell line	EGCG	Induction of apoptosis through proteasome inhibition
Thangapazham *et al*.	MDA-MB-231 cell line	EGCG	Cell cycle regulation

proliferation, viability and cell death of MCF-7 cells. MCF10A cells were treated with 0, 0.5, 2.5, 10, 40, and 100 μg/mL of GTC for 48 h. The data indicated cytotoxic activity of green tea catechins at 100 μg/mL, but not at 0.5, 2.5, 10, and 40 μg/mL, in reducing cell viability, inhibition of cell proliferation or induction of apoptotic cell death. Trying to investigate the mechanisms of actions of green catechins, the authors used cDNA microarrays to detect differentially regulated genes that were changed in carcinogen-treated cells but whose changes were suppressed by green tea catechins. Initially, the investigators identified the genes whose expressions were changed in carcinogen-treated cells compared to their counterpart expression levels in untreated cells. After gene expression data analysis, the authors identified 479 differentially expressed genes in carcinogen-treated cells, versus expression in untreated, counterpart cells. Subsequently, the authors identified genes associated with ROS elevation, cell proliferation, the ERK pathway activation, and DNA damage, that were not induced in green tea catechin and carcinogen-treated cells and concluded that BAX, COX17, and MRPL41 associated with ROS elevation, B4GALT1, BARHL1, BOLA3, and MT1E associated with cell proliferation, S100P and SPRR1B associated with ERK pathway activation, and ATM and PER1 associated with DNA damage were up-regulated in carcinogen-treated cells but were suppressed in green tea catechin and carcinogen-treated cells. In addition, TNFRSF8, a gene associated with negative regulation of cell proliferation was down-regulated in carcinogen-treated cells but not down-regulated in GTC- and carcinogen-treated cells. In addition PCR method was applied to validate cDNA data by arbitrarily choosing a gene from each category. PCR data were in agreement with cDNA data . In an effort to further investigate the mechanism of the protective effect of green tea catechins, the authors showed that short term exposure of human breast epithelial MCF10A to NNK and B[a]P induced transient ROS elevation leading to ERK pathway activation, cell proliferation and chromosomal DNA damage, effects that were blocked by non toxic concentrations of green tea catechins [7-9].

Bigelow and Cardelli [10] have investigated the effect of EGCG on inhibition of hepatocyte growth factor (HGF) signaling in the immortalized, nontumorigenic breast cell line, MCF10A. HGF treatment induced rapid, sustained activation of Met (HGF receptor), ERK1/2 and AKT pathways. Pretreatment of cells with concentrations of EGCG as low as 0.3 μm inhibited HGF-induced Met phosphorylation and downstream activation of AKT and ERK. Treatment with 5.0 μm EGCG blocked the ability of HGF to induce cell motility. In order to determine if EGCG can block HGF signaling at the level of Met receptor activation, MCF10A cells were pretreated for half an hour with increasing concentrations of EGCG (0.07-20 μm). HGF (30 ng/ml) was added in the presence of EGCG for 15 min and protein lysates were prepared. Western blot analysis revealed that concentrations of EGCG as low as 0.07 μm EGCG partially blocked Met activation, while concentrations of 0.3 μm and above completely blocked phosphorylation of Met, Erk and Akt. However, (-)-epicatechin was unable to inhibit HGF-induced events at any concentration tested. On the other hand, (-)-epigallocatechin, completely repressed HGF-induced AKT and ERK phosphorylation at concentrations of 10 and 20 μm, but was incapable of blocking Met activation. Despite these observations, EGC did inhibit HGF-induced motility in MCF10A cells at 10 μm [10].

Modulation of Molecular Chaperones

Tran *et al*. [11] have shown that the anti-tumor activity of EGCG in MCF-7 breast cancer cells is mediated by the inhibition of HSP70 and HSP90. In that study, MCF-7 cells were cultured in the presence or absence of EGCG for 24h. The effect of EGCG on MCF-7 human breast cancer cells was examined by increasing concentrations of EGCG and cell proliferation assay was performed. Treatment with EGCG (10 ~ 200 μM) inhibited the growth of MCF-7 cells in a dose-dependent manner with an IC_{50} of 150 μM with extensive inhibition of cell growth being observed in the cells treated with high concentration (200 μM) of EGCG. Cell cycle distribution analysis showed that the cells were mainly arrested at the G2/M phase. In addition, cell colony formation was evaluated by a soft agar assay. MCF-7 cells were plated on a soft agar matrix, treated with EGCG, and incubated at 37°C for 10 days. EGCG inhibited colony formation in a dose-dependent manner. MCF-7

cells were incubated with increasing concentrations of EGCG (10 ~ 200 μM) for 24 h. The levels of protein and mRNA of HSP70 and HSP90 were decreased by 100 μM of EGCG, while the other HSPs (HSP110, HSP60, HSP40, and HSP27) were unaffected. Transcriptional activity of HSP70 and HSP90 was determined by luciferase reporter assay. EGCG suppressed the expression of HSP70 and HSP90 *via* inhibition of the promoter activity of HSP70 and HSP90. In addition the interaction between the HSPs and EGCG was assessed using EGCG-conjugated Sepharose 4B beads and it was confirmed that both HSP70 and HSP90 interact efficiently with EGCG. Furthermore, the competition between EGCG and ATP for the ATPase binding pockets of HSP70 and HSP90 was examined and it was shown that EGCG competes with ATP for binding to the ATP binding pocket of HSP70 and HSP90 in human breast cancer cells. [11].

APOPTOSIS INDUCTION

Redox Modulation and Apoptosis

Green tea catechins are known to have both pro-oxidant and antioxidant properties. Catechins possess free radical scavenging properties and act as biological antioxidants. In addition, treatment of experimental animals with green tea catechins increases endogenous antioxidants such as glutathione peroxidase and reductase, superoxide dismutase and catalase [12,13]. However, in the context of breast cancer, the effect of green tea catechins on antioxidant defense system i.e glutathione and enzymes has not been investigated. In fact, two studies have suggested that the anticancer effect of green tea catechins is due to the induction of apoptosis through redox modulation. These studies have shown that green tea catechins possess free radical scavenging and pro-oxidant properties. Although not investigated in these studies, pro-oxidant properties of green tea catechins are anticipated to up-regulate endogenous antioxidants.

In the trial of Kaur *et al*. [14], the effect of green tea catechins was investigated in the of C3(1) SV40 T,t antigen transgenic multiple mammary adenocarcinoma (TAg) mouse model. Female Tag mice develop mammary tumours characterised by inactivated tumour suppressor genes p53 and Rb, thus mimicking hormone receptor negative human breast cancer. Mice (10 per group) were treated with 0.05% green tea catechins as the sole source of drinking fluid for 25 weeks. Treatment delayed carcinogenesis as evidenced by a significant decrease in volume and size of tumors in the mice exposed to green tea extract. The authors suggested that green tea catechins affected carcinogenesis through redox modulation and induction of apoptosis. The hypothesis of redox modulation was supported by experimental data indicating that changes in cancer parameters correlated with tea-polyphenol mediated decreases in the levels of a malonyldialdehyde-DNA adduct in the tumors. The hypothesis of apoptosis induction was supported by the increased tumor levels of cleaved caspase 3 protein that were identified in the tumors [14].

Hsuuw *et al*. [15] have demonstrated that treatment of human MCF-7 cells with 50 μM EGCG can induce apoptotic changes, including mitochondrial membrane potential changes and activation of c-Jun N-terminal kinase (JNK), caspase-9, and caspase-3 while higher concentrations of EGCG (100-400 μM) do not induce apoptosis, but rather trigger necrotic cell death in MCF-7 cells. In that study, MCF-7 cells were incubated with various doses of EGCG for 6 h, and cell viability was determined with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay. For assessment of apoptosis, oligonucleosomal DNA fragmentation in apoptotic cells was measured using the TUNEL assay. Cell apoptosis and necrosis were also assessed by propidium iodide and Hoechst 33342 staining. Necrosis was also assessed by measurement of the lactate dehydrogenase activity present in the culture medium. In addition, reactive oxygen species generation was assessed using the 2_-7_-dichlorofluorescein diacetate (DCFDA) dye and determined using a fluorescence ELISA reader. The data indicated that 10 to 400 μM EGCG treatment resulted in dose-dependent decrease in cell viability. Trying to elucidate the mechanism, the authors have shown that treatment of MCF-7 cells with lower concentrations of EGCG (10-50 microM) directly increased intracellular oxidative stress, while higher concentrations (100-400 microM) did not. In addition, low concentrations (10-50 microM) of EGCG treatment, resulted in loss of mitochondrial membrane potential while higher concentrations (100-400 microM) had no such effect. Treatment of MCF-7 cells with 10-50 μM EGCG led to activation of JNK, caspase-9, and capase-3 while treatment of these cells with 100-400 μM EGCG had little effect on these apoptotic changes. On the other hand, caspase 8 was not activated by either treatment, indicating in not involved in EGCG induced cell death. The authors further investigated if Fas receptor was involved in EGCG induced apoptosis or necrosis. The data indicated no change in Fas expression levels following low or high dose EGCG treatment of MCF-7 cells. It was subsequently shown, that treatment of MCF-7 cells with 10-50 microM EGCG causes increases in Bax protein levels and decreases in Bcl-2 protein levels, shifting the Bax-Bcl-2 ratio to favour apoptosis, while treatment with 100-400 microM EGCG has no such effect. In addition, the investigators examined changes in cellular content of ATP, following high and low dose EGCG treatment of MCF-7 cells. The data indicated a dose-dependent decrease in intracellular ATP levels in cells treated with high-dose EGCG, while no such effect was observed in cells treated with low dose EGCG. The apoptotic cascade involved JNK activation, Bax expression, mitochondrial membrane potential changes, and activation of caspase-9 and caspase-3 [15].

Downregulation of Telomerase and Apoptosis

The telomeres are 15-20 kb long segments of hexameric repeats of 5'-TTAGGG-3', that protect the ends of human chromosomes from the degradation associated with cell division. In normal cells telomeres lose up to 300 bp of DNA per cell division that ultimately leads to senescence; however, most cancer cells bypass this lifespan restriction through the expression of telomerase. Three studies have demonstrated that green tea catechins induce apoptosis in breast cancer cells through down-regulation of telomerase *via* epigenetic mechanisms. Yet, these are the only studies that suggest modulation of epigenetic processes by green tea catechins in the context of breast cancer.

Mittal *et al*. [16] have demonstrated that green tea catechins induce apoptosis in MCF-7 breast cancer cells through downregulation of telomerase. The authors have shown that treatment with EGCG dose-dependently inhibited (20-100%) the reproductive or colony forming potential, and also decreased cell viability at different time points studied (approximately 80% inhibition) in human breast carcinoma MCF-7 cells. In addition, treatment with EGCG for 48 and 72 h markedly increased the percentage of apoptotic cells (32-51%) in MCF-7 cells compared to that of non-EGCG treated cells (8-14%). In addition, the authors attempted to elucidate the mechanism of decreased cell viability and induction of apoptosis in breast carcinoma cells by EGCG and they showed that treatment of MCF-7 cells with EGCG dose-dependently inhibited telomerase activity, and also inhibited the mRNA expression of hTERT (human telomerase reverse transcriptase), the catalytic subunit of telomerase. The same investigators also demonstrated that EGCG also inhibited the protein expression of hTERT, which indicated that inhibition of telomerase was associated with down-regulation of hTERT [16].

Belretch *et al*. [17] reported that treatment of MCF-7 cells with EGCG resulted in decreased hTERT mRNA expression. In that study, exposure to EGCG reduced cellular proliferation. MCF-7 cells were treated with 100 μM EGCG. Cells were counting using

tryptan blue staining and morphological data were obtained at 3 day intervals. By day 6 MCF-7 cells showed a significant reduction in cell growth (of about 88%). Apoptosis was measured using fluorescent labeling and fluorescent activated cell sorting (FACS). At day 9 MCF-7 cells showed high levels of apoptosis. Thus, the authors investigated if the reduction of apoptosis was modulated by downregulation of hTERT. It was subsequently shown that at 6th day of exposure to 100 μM EGCG, hTERT gene expression was reduced by23% when compared with the untreated control grown in the absence of EGCG in the same time period. Furthermore, down-regulation of hTERT gene expression in MCF-7 cells appeared to be largely due to epigenetic alterations as evidenced by the time-dependent decrease in hTERT promoter methylation and ablated histone H3 Lys9 acetylation [17].

Meeran *et al*. [18] have shown that treatment with EGCG dose- and time-dependently inhibited the proliferation of human breast cancer MCF-7 and MDA-MB-231 cells. MCF-7, MDA-MB-231 cells as well as normal control human breast MCF10A cells were treated with 20-60 μM EGCG for 3, 6, 9 and 12 days for cell growth assay. Doses of up to 60 μM of EGCG had negligible cell proliferation inhibition activity in control MCF10A cells while the same doses inhibited cellular proliferations for MCF-7 and MDA-MB-231 cells. In addition, significant levels of apoptosis were observed at 9 and 12 days with 40 μM EGCG treatment for both MCF-7 and MDA-MB-231 cells. Furthermore, treatment with EGCG (40 μM) time dependently inhibited hTERT expression in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 cells at 9 and 12 days of treatment. The inhibition of the transcription of hTERT was mediated through epigenetic mechanisms. EGCG (40 μM) inhibited the activities of DNMTs (DNA methyl transferases) and HATs (histone acetyl transferases) at 6 and 9 days of treatment in human breast cancer cells. However, EGCG treatment had no effect on HDAC (histone deacetylase activity). Since the hTERT promoter is hypermethylated in most cancer cells for its transcriptional activation, the authors assessed the methylation status of the *hTERT* promoter region from −288 to −31 covering 26 CpG dinucleotides and various overlapping transcription factor binding sites. Bisulfite-sequencing was used to detect the *hTERT* methylation patterns of EGCG treated human breast cancer cells. It was shown that control untreated MCF-7 and MDA-MB-231 breast cancer cells maintained a high level of methylation at promoter sites at 87.6±3.24 % and 79.4±2.29 %, respectively, whereas treatment with EGCG (40 μM) considerably reduced promoter methylation in a time-dependent manner. Thus, the authors concluded that the downregulation of hTERT expression was due to hTERT promoter hypomethylation and histone deacetylation, mediated at least partially through inhibition of DNA methyltransferase and histone acetyltransferase activities, respectively [18].

Directly Targeting Apoptotic Cascade

In another study, Alshatwi [19] has shown that the green tea catechin, catechin hydrate reduces proliferation of MCF-7 cells in a dose and time dependent manner through induction of extrinsic and intrinsic apoptotic pathways as evidenced by up-regulation of the expression of pro-apoptotic genes such as caspase-3, -8, and -9 and TP53 [19]. The authors attepted to investigate whether apoptosis was the mode of death for MCF-7 cells treated with catechin hydrate. For concentration and time dependent studies, two sets of CH concentrations (50 μg/mL and 150 μg/mL; 300 μg/mL and 600 μg/mL) were considered for treatment of MCF-7 cells for 24 hours. The authors found that 50 μg/mL CH did not show any significant induction of apoptosis whereas 600 μg/mL CH completely killed the cells. Thus, 150 μg/mL and 300 μg/mL concentrations of CH were used for further studies. Cell viability was assayed using a trypan blue exclusion test. Cells were treated with either 150 μg/mL or 300 μg/mL catechin hydrate for 24, 48 and 72 hours for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. In addition, the cells were incubated with either 150 μg/mL or 300 μg/mL catechin hydrate for 24 and 48 hours for real-time quantitative PCR analysis. The viability assay resulted in cell viability greater than 95%. MCF-7 breast cancer cell proliferation was dose and time dependently inhibited by catechin hydrate treatment. In that study, cells were exposed to 150 μg/ml CH and 300 μg/mL CH for 24 hours, resulting in 40.7% and 41.16% apoptotic cells, respectively. In addition, a 48-hour exposure to 150 μg/ml CH and 300 μg/ml CH resulted in 43.73% and 52.95% apoptotic cells, respectively. Interestingly, after 72 hours of exposure to both concentrations of CH, almost 100% of cells lost their integrity. Further mechanistic investigation confirmed increased expression of caspase-3,-8, and -9 and TP53 in a time-dependent and dose-dependent manner, as determined by real-time quantitative PCR.

In addition, Roy *et al*. [20] have shown that EGCG induced apoptosis in MDA-MB-468 cells through direct modulation of pro-apoptotic genes and anti-apoptotic genes. MDA-MB-468 cell line, used in that study, is an established *in vitro* model for estrogen receptor negative breast cancer. Outcomes investigated included the antiproliferative effect of EGCG on MDA-MB-468 cells measured by colony forming assay; the cell growth and viability of EGCG cells under EGCG treatment. Furthermore, EGCG induced apoptosis in MDA-MB-468 cells was determined by DNA ladder assay and by fluorescent staining. In addition the quantative measurement of apoptotic cell death caused by EGCG treatment was done by flow cytometry. Treatment with 5-80 microg/mL EGCG resulted in dose-dependent and time-dependent (24-72 hours) inhibition of cellular proliferation and cell viability in MDA-MB-468 cells. Interestingly, treatment of MDA-MB-468 cells with ≥40 μg/mL of EGCG almost completely inhibited colony formation capacity. Significant inhibition in cell viability was observed with the treatment of 20 to 80 μg/mL concentrations of EGCG at 24, 48, and 72 hours time points. Thus, doses of EGCG at 20, 40 and 60 μg/mL were used in the subsequent mechanistic studies. The observed decrease in cell viability was associated with the induction of apoptosis. 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, while similar results were obtained after 24 and 72 hours of EGCG treatment although DNA damage was more intense after 72 hours of treatment. EGCG-induced apoptosis in MDA-MB-468 cells was subsequently morphologically identified using fluorescence staining with Hoechst 33342. MDA-MB-468 cells were treated (a) in the presence or absence of EGCG (20, 40 and 60 μg/mL) for 48 hours and (b) in the presence or absence of EGCG (20, 40 and 60 μg/mL) for 72 hours. Fluorescence microscopy data indicated that EGCG treatment dose- and time-dependently induced apoptosis in breast cancer cells. In addition, the number of apoptotic cells was found 18% to 30% after 48 hours of EGCG treatment (20-60 μg/mL), while 72 hours of EGCG (20-60 μg/mL) treatment resulted in 20% to 54% apoptotic cells.

Subsequent mechanistic studies showed that EGCG treatment led to increased expression of tumor suppressor protein p53 and its phosphorylation at Ser 15 residue. Specifically, treatment of MDA-MB-468 cells with EGCG (20, 40, and 60 μg/mL) for 48 and 72 hours resulted in dose dependent increased in the expression of protein p53. Further use of a phospho-specific antibody against p53 at Ser 15 residue followed by Western blot analysis indicated that that the level of phosphorylation of p53 was increased by 2.0-to2.5-fold after 48 hours of EGCG (20-60 μg/mL) treatment to MDA-MB-468 cells. To further examine the mechanism of antiproliferative effect of EGCG on MDA-MB-463 cells, the authors hypothesized that 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. Indeed, it was found that EGCG treatment (20, 40, and 60 μg/mL) for 48 and 72 hours decreased the expression of antiapoptotic protein Bcl-2, associated with the inhibition of apoptosis but increased the expression of proapoptotic protein Bax, associated with the induction of apoptosis. The hypothesis that the induction of apoptosis in MDA-MB-468 cells by EGCG was mediated through reduction in antiapoptotic protein Bcl-2 and increased expression of proapoptotic protein Bax and their ratio was further supported by data indicated that the ratio of Bax/Bcl-2 was dose-dependently increased both at 48 and 72 hours after EGCG treatment. The authors further suggested that the increased ratio of Bax/Bcl-2 proteins resulted in increased release of cytochrome c from mitochondria into cytosols, increased expression of apoptotic protease activating protein 1, and activation of caspase-3 and poly(ADP-ribose) polymerase, leading to apoptosis in MDA-MB-468 cells [20]. Thus, the effect of EGCG on the expression of these proteins was further investigated by Western blot analysis. Western blot data indicated that EGCG treatment of MDA-MB-468 cells for 48 and 72 hours resulted in dose-dependent increase in cytochrome *c* release from mitochondria, induction of apoptotic protease activating protein 1, activation of caspase 3 and PARP proteins.

Vergote *et al*. [21] have shown that EGC induces apoptosis in MCF-7 and MDA-MB-231 cells through FAS signalling. EGC strongly inhibited the growth of breast cancer cell lines MCF-7 and MDA-MB-231 without affecting the growth of normal breast epithelial cells. The inhibition of breast cancer cell growth was due to an induction of apoptosis, without any change in cell cycle progression. This suggestion is based on experimental data showing, (1) that neutralizing antibodies against the death receptor Fas and inhibitors of caspases, such as caspase-8 and -10, inhibit the EGC-triggered apoptosis; (2) EGC treatment was correlated with a decrease in Bcl-2 and an increase in Bax level, based on immuno-blotting data (3) Given that MCF-7 cells express a wild-type p53 whereas MDA-MB-231 cells express a mutated p53, EGC induction of apoptosis in both these cell lines suggests that the EGC-triggered apoptosis was independent of p53 status [21].

Induction of Apoptosis Through Proteasome Inhibition

Landis Piwowar *et al*. [22] have shown that EGCG treatment inhibits proteasome in MDA-MB-231 cells leading to accumulation of proteasome target proteins and subsequent induction of apoptosis. MDA-MB-231 cells were treated with 50 μmol/L EGCG every 24h for 72 h using DMSO as a control. The observed proteasome inhibition from EGCG treatment correlated with the accumulation of ubiquitinated proteins, ubiquitinated IκB-α, and other proteasome target proteins, p27 and Bax and induced a 50% reduction in cell proliferation. Furthermore, treatment led to induction of apoptosis, as evaluated by morphologic changes indicative of apoptotic cell death, i.e. the presence of apoptotic nuclei, the event of activated caspase-3/caspase-7, and, production of the cleaved PARP fragment.

CELL CYCLE REGULATION

In addition, it has been shown that green tea catechins reduce cell proliferation by modulation of cell cycle progression. Thangapazham *et al*. [23] have shown that green tea polyphenols (60 μg/ml) and EGCG treatment (50-80 μg/ml) for 24h had the ability to arrest the cell cycle at G1 phase of human breast cancer MDA-MB-231 cells as assessed by flow cytometry. Both green tea polyphenols and EGCG were pro apoptotic as showed by intranucleosomal cleavage of DNA, which was visualized by a typical DNA ladder pattern. Induction of apoptosis was primarily due to Bax/bcl2 ratio favouring apoptosis, to the upregulation of proteases and to PARP cleavage. Western blot analysis of cell cycle proteins of MDA-MB-231 cells treated with 50 and 80 mg/ml of EGCG and 40 and 60 mg/ml green tea polyphenols for 24 h showed that EGCG and green tea polyphenols increased bax and decreased bcl2 in a dose dependent manner. In addition, under the same experimental conditions, the full length form of the PARP protein, a substrate for caspase-3, degraded ito the cleaved form. Furthermore, the expression of Cyclin D, Cyclin E, CDK 4, CDK 1 and PCNA were down regulated over the time in 40 and 60 mg/ml green tea polyphenol and 50 and 80 mg/ml EGCG treated experimental group, compared to the untreated control group as evaluated by western blot analysis for cell cycle proteins, which corroborated the G1 block [23].

SUGGESTIONS FOR FURTHER RESEARCH

Evidence suggests that green tea catechins block breast cell carcinogenesis and inhibit cell growth and proliferation of human breast cancer lines [24-26]. Although a number of mechanisms have been proposed, the mode of action of green tea catechins is not clearly defined. Mechanistic studies investigate different mechanisms, rendering it difficult to deduce the molecular links among the different putative targets of green tea catechins. It is commonly difficult to know if the reported experimental effects associated with treatment with green tea catechins are due to their direct or indirect effects. It could be suggested that the redox regulation or the modulation of epigenetic alterations are the primary effects of green tea catechins leading to regulation of cell cycle, cell survival and apoptosis through modulation of gene transcription. Although this suggestion seems justified for multitarget agents such as green tea catechins, it is definitely not supported from existing evidence. Although experimental evidence suggests that green tea catechins modulate redox [27] and epigenetic processes, relevant data are limited in the context of breast cancer. Most experimental trials investigate the effect of green tea catechins on a specific target without taking into account the other putative targets of green tea catechins. It would be interesting if future experimental trials could take into account that green tea catechins are multi-target agents and attempt to link every novel proposed target with the other already proposed targets of green tea catechins. Although such an approach would be probably experimentally demanding, it would contribute knowledge not only on the mechanism of anti-proliferative action of green tea catechins but also on novel targets of breast cancer treatment.

However, although the molecular links among the proposed mechanisms of action of green tea catechins are often missing, it must be emphasized that all the proposed mechanisms indicate that green tea catechins inhibit growth and /or promote apoptosis. Thus, it can be concluded that basic research data indicate that green tea catechins would be promising multi-target agents in the chemo-prevention, adjuvant and metastatic treatment of breast cancer. Given the anticipated favourable safety profile of green tea catechins, further investigation of the efficacy and safety of green tea catechins in the clinical setting of breast cancer treatment and/ or chemoprophylaxis seems scientifically justified.

To our knowledge, up to now, there is only one publication of a phase IB randomized, double-blinded, placebo-controlled, dose escalation study of polyphenon E in women with hormone receptor-negative breast cancer [28]. In that study, forty women were randomized: 10 to placebo, 30 to Polyphenon E (16 at 400 mg, 11 at 600 mg, 3 at 800 mg). There was one dose limiting toxicity at 400 mg (grade III rectal bleeding), three dose limiting toxicities at 600 mg (grade II weight gain, grade III indigestion and insomnia), and one dose limiting toxicity at 800 mg (grade III liver function abnormality) [28]. Undoubtedly, further investigation is warranted.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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