Epigallocatechin gallate induces the steady state mRNA levels of pS2 and PR genes in MCF-7 breast cancer cells

Mohan C Manjegowda, Gauri Deb & Anil M Limaye*

Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781 039, India

Investigations using *in vitro* and *in vivo* models of breast carcinogenesis have demonstrated anti-neoplastic activity of the green tea polyphenol, epigallocatechin gallate (EGCG). Although a number of molecular targets of EGCG have been identified, its impact on the expression of estrogen target genes is not completely understood. Here, we examined the mRNA expression levels of two estrogen target genes, namely Trefoil Factor 1 (pS2) and Progesterone Receptor (PR) in MCF-7 cells treated with EGCG. We observed that treatment with 40 μM EGCG, which caused only 20% decrease in cell viability, resulted in increased steady state expression levels of pS2 and PR mRNA. This suggests that EGCG may exert its biological activities, at least in part, by influencing the expression of estrogen target genes.

Keywords: Breast cancer, Carcinogenesis, Epigallocatechin gallate, Estrogen responsive markers, MCF-7, Progesterone receptor, Trefoil Factor 1 (pS2)

Studies on chemopreventive and antitumor activities of green tea polyphenols are not uncommon[1-3]. Epigallocatechin gallate (EGCG), the major green tea polyphenol in particular, has been a subject of global interest as a potential chemopreventive or chemotherapeutic supplement for breast cancer. While on one hand epidemiological studies suggest an inverse correlation between green tea consumption and breast cancer risk[4-6], investigations using cell culture and animal models of breast carcinogenesis on the other hand have demonstrated antiproliferative, antitumor, anti-invasive and anti-metastatic properties of EGCG[7-10]. Mechanisms of how EGCG affects cell proliferation, apoptosis, migration and cell cycle by manipulating the function of a wide range of molecular targets have also been studied[8-9,11-13]. However, the question as to how EGCG affects estrogen responsive genes has not been addressed. This issue is of relevance to our notion of EGCG as a potential chemopreventive or chemotherapeutic agent against breast cancer, which is estrogen dependent in majority of the newly diagnosed cases. In the present study, using estrogen receptor α (ERα) positive MCF-7 breast cancer cells as a model, we examined the effect of optimized concentration of EGCG on the steady state mRNA expression levels of trefoil factor 1 (pS2) and progesterone receptor (PR), the known estrogen responsive markers.

Materials and Methods

Cell culture materials, reagents and chemicals— Tissue culture flasks (25 cm²), 35 mm dishes and 96-well plates were purchased from Greiner Bio-One, Germany. DMEM-F12 (with and without phenol red) was from Invitrogen Corporation, USA. Fetal Bovine Serum (FBS) was purchased from PAA Laboratories, GmbH, Austria. Charcoal-stripped FBS was purchased from Hyclone, Thermo Scientific, USA. Antibiotic Solution (100X) and trypsin-EDTA were from HiMedia, Mumbai, India. Propidium Iodide (PI), Nonidet P-40 (NP-40) and (-)epigallocatechin-3-gallate (EGCG) were purchased from Sigma-Aldrich, USA. Other salts, solvents and buffer components used in this study were from Merck, Mumbai, India or SRL, Mumbai, India.

Cell culture and treatments—MCF-7 cells were routinely cultured in 25 cm² flasks using M1 medium (phenol red containing DMEM-F12, 10% heat inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin) under standard culture conditions of 37°C and 5% CO_2. Confluent cells were trypsinized, and either seeded in 96 well plates (7×10^3 cells per well) or 35 mm dishes (1.5×10^5 cells per dish) in M1 medium depending on the experiment. However, treatment of

_____
*Correspondent author
Telephone: +91-0361-258 2218
E-mail: amul@iitg.ernet.in

cells with EGCG were carried out in M2 medium (phenol red-free DMEM-F12, 10% charcoal-stripped and heat inactivated FBS, 100 units/ml penicillin and 100 µg/mL streptomycin).

For MTT assays, MCF-7 cells were seeded with M1 medium in a 96-well plate and incubated for 36 h in a CO_2 incubator maintained at 37^oC and 5% CO_2. The medium was then replaced with M2 medium and further incubated for 2 h. The experiment was then started by replacing the spent M2 medium in groups of wells with fresh M2 medium containing increasing concentrations of EGCG, from 2 to 200 µM. Wells containing M2 medium treated with ethanol (vehicle used for making EGCG stock solution) alone served as controls. The cells were treated for 72 h before proceeding with MTT assay.

In other experiments involving flow-cytometry or analysis of mRNA expression levels, MCF-7 cells were seeded in 35 mm dishes with M1 medium followed by replacement with M2 medium after 36 h. After 2h of incubation, the spent M2 medium was replaced with fresh M2 medium containing the indicated concentrations of EGCG. Cells treated with ethanol alone served as controls. The cells were treated for 24 or 72 h before proceeding with flow-cytometry or semi-quantitative RT-PCR, respectively.

MTT assay—After treatment of cells for the stipulated period of time, the spent medium was aspirated out. The cells were washed with phosphate buffered saline (PBS). Each well was flooded with 100 µL of MTT reagent (0.5 mg/mL MTT in DMEM-F12) and incubated at 37^oC and 5% CO_2. After 3 h, the MTT reagent was removed and the formazan crystals were dissolved in 100 µL of DMSO. The absorbance was measured at 570 (A_{570nm}) and 690 nm (A_{690nm}) using a multimode reader. The difference between the two absorbance values ($A_{570nm}-A_{690nm}$) was determined as a measure of cell viability. The $A_{570nm}-A_{690nm}$ values obtained for EGCG treated cells were expressed as percent of those obtained for untreated control (% viability).

Flow-cytometry—Hypotonic PI staining solution (20 µg/mL PI in 0.1% trisodium citrate dihydrate solution containing 0.03% NP-40) was added to the culture dishes. Cells were dislodged and lysed to release their nuclei by repeated pipetting. Nuclei, along with cell debris, were transferred into a fresh tube and pelleted by centrifugation at 300 g for 5 min at 4^oC. Pellet was re-suspended in fresh hypotonic PI staining solution and analyzed by flow cytometry

(FACSCalibur, Becton, Dickinson, USA). PI stained nuclei were analyzed in FL2 channel (585/42 band pass filter), and a dot plot of FL2-area *vs.* FL2-width was used to discriminate doublets. Histogram of FL2-area for gated population was plotted for visual representation of cells in different stages of cell cycle.

Semi-quantitative RT-PCR—Total RNA was isolated from cells using TRIzol (Invitrogen Corporation, USA) according to manufacturer's instructions. Contaminating DNA in the total RNA was digested by DNase I (New England Biolabs Inc, USA) treatment for 20 min at 37^oC in DNase I digestion buffer supplied by the manufacturer. The total RNA was then cleaned using RNeasy mini kit (Qiagen Sciences Inc, USA). Equal quantities (1 or 2 µg) of total RNA from control and treated cells were reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Invitrogen Corporation, USA) in a total reaction volume of 20 µL. The resultant cDNAs were diluted five times and 2 µl was used as template for PCR reaction with gene specific primers for pS2, PR and β-2 microglobulin (β2M). The number of cycles of amplification required for product formation in the linear range of detection by agarose gel electrophoresis was determined separately for each gene. The primer sequences along with their annealing temperatures, number of cycles used and amplicon length have been listed in Table 1. The PCR products were electrophoresed on 2% agarose gels and the images of the ethidium bromide stained bands were captured using Gel Logic 1500 imaging system (Carestream Health Inc, USA). The images were processed and analyzed using the ImageJ software which was downloaded from the NCBI website[16]. The band intensities for each gene were normalized against β2M. The normalized intensities for pS2 and PR in controls were assigned the value of 1 and those obtained for treated cells were expressed as fold change over control.

Table 1—Primers used for semi-quantitative RT-PCR

Gene	Primer sequences (5'→ 3')	Annealing temperature (^oC)	No. of cycles	Amplicon size (bp)
β2M	F: GTCTTTCAGCAAGGACTGG R: GCATCTTCAAACCTCCATG	52	30	161
pS2	F: AATGGCCACCATGGAGAACA R: ATAGAAGCACCAGGGGACCC	56	27	211
PR	F: CGCGCTCTACCCTGCACTC R: TGAATCCGGCCTCAGGTAGTT	58	35	121

pS2 and PR primers were from Banerjee *et al.*[14] and Bieche *et al.*[15], respectively.

Statistical analysis—For MTT assay, the % cell viability values for control and treatment groups were represented as graphs plotted in Microsoft Excel. ANOVA for the group means followed by Tukey's test for comparison of pairs of group means was performed using R statistical package. For the analysis of semi-quantitative RT-PCR, data is represented as mean fold change ± SD. Statistical analysis of the mean fold change in pS2 and PR mRNA expression in treatment group *vs.* control was performed using the Student's *t*-test (unpaired, one-tailed) in MS Excel. In all statistical tests, $P < 0.05$ was considered significant.

Results

The MTT assay demonstrated a dose dependent decreased viability in MCF-7 cells treated with increasing concentrations of EGCG from 2 to 200 µM (Fig. 1). The concentration of EGCG required to decrease viability by 50% of control was approximately 70 µM. In our experiment, 40 µM EGCG, which was the minimum concentration required to observe a significant effect, reduced the cell viability by 20%. All concentrations of EGCG from 40 µM onwards significantly reduced cell viability compared to control (n=12, $P<0.0001$, Fig. 1).

In flow-cytometry, the profiles of G1, S and G2 cell populations were similar in control and 40 µM EGCG treated cells (Fig. 2A, left and middle panel). However, cells treated with 200 µM EGCG showed a markedly different profile (Fig. 2A, right panel) with a significant increase in cell debris indicating severe cytotoxicity of MCF-7 cells. This is consistent with our microscopic observation that the control and 40 µM EGCG treated cells had normal cell
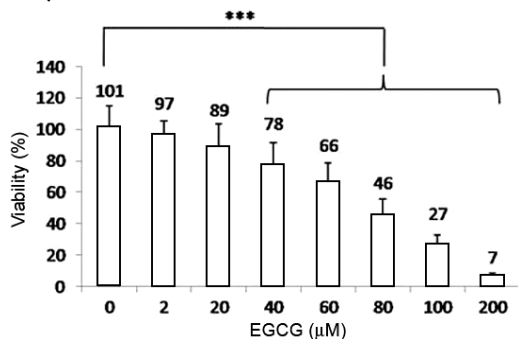
Fig. 1— Dose dependent effect of EGCG on MCF-7 cell viability. MCF-7 cells (7000 cells/well) were treated with indicated concentrations of EGCG for 72 h and cell viability was determined by MTT assay. Bars represent mean % viability ± SD (n=12, *** $P < 0.0001$). The number above each bar is the average % viability.

morphology while 200 µM EGCG treated cells showed clear signs of cytotoxicity (Fig. 2B). Also, cells treated with 200 µM EGCG yielded low amounts of partially degraded total RNA compared to the control and 40 µM EGCG treated cells (Fig. 2C).

On the basis of the above results, we chose to examine the effect of 40 µM EGCG on mRNA expression levels of pS2 and PR, the two estrogen induced genes, using β2M mRNA level as the internal control. As shown in Fig. 3, MCF-7 cells treated with 40 µM EGCG for a period of 72 h showed significantly higher levels of pS2 and PR mRNA with respect to vehicle treated controls. The fold increments observed in the steady state mRNA levels of pS2 and PR were 3.05±0.95 (n=3, p=0.032) and 3.53 ±1.02 (n=3, p=0.025), respectively.

Discussion

Effects of EGCG on cell viability and gene expression have been extensively reported in literature over a wide range of EGCG concentrations (0.1-200 µM). However, to study the effect of EGCG treatment on pS2 and PR expression, an appropriate concentration that would not severely compromise cell viability was chosen to ensure that the observed modulation of pS2 and PR mRNA levels was not a collateral effect of severe cytotoxicity. In our MTT assay, a modest (20%) but significant reduction of MCF-7 cell viability was observed at 40 µM EGCG. This is in contrast to a significant reduction of 93% in 200 µM EGCG (Fig. 1) which could be largely due to severe cytotoxicity as indicated by flow-cytometric profile (Fig. 2). Hence, we conclude that the observed induction of pS2 and PR mRNA at 40 µM EGCG is due to its effect on gene expression.

There have been reports available which are either consistent or contradictory to our observation of PS2 and PR induction by EGCG. Goodin *et al.*[17] demonstrated specific binding of EGCG to both ERα and ERβ and showed that EGCG can evoke ER mediated transcriptional induction of reporter genes. In an independent study, Kuruto-Niwa *et al.*[18] have shown that at sub-micromolar concentrations, EGCG synergizes with estrogen to induce the estrogen response element (ERE)-driven reporter gene activity, indicating that the EGCG can evoke estrogenic responses at the level of gene expression. Consistently, we have demonstrated that 40 µM EGCG can induce the mRNA expression levels of pS2 and PR (Fig. 3). Contrary to our observation,

Farabegoli *et al.*[19] and recently, De Amicis *et al.*[20] have reported down-modulation of pS2 mRNA in MCF-7 cells treated with 100 µg/ml EGCG (>200 µM) and 40 µM EGCG, respectively. In our study, we have shown that 200 µM EGCG induces severe cytotoxicity, disrupts the cellular integrity and yields degraded RNA (Fig. 2). Hence, down-modulation of pS2 at 200 µM EGCG may not be a true reflection of EGCG action at the level of gene expression. It should also be noted that the method of EGCG treatment was different in our study compared

to De Amicis *et al.*[20]. While we performed EGCG treatment for 72 h with medium change every 24 h, De Amicis *et al.* treated the cells with EGCG for 24 h after 48 h of serum starvation. The 48 h serum starvation might have compromised the viability of the cells to a great extent before they were exposed to 40 µM EGCG. In an independent study, EGCG in combination with trichostatin A (TSA) has been shown to cause reactivation of ERα in the ER negative MDA-MB-231 cells via a mechanism which involves chromatin remodeling at the ERα promoter[21].
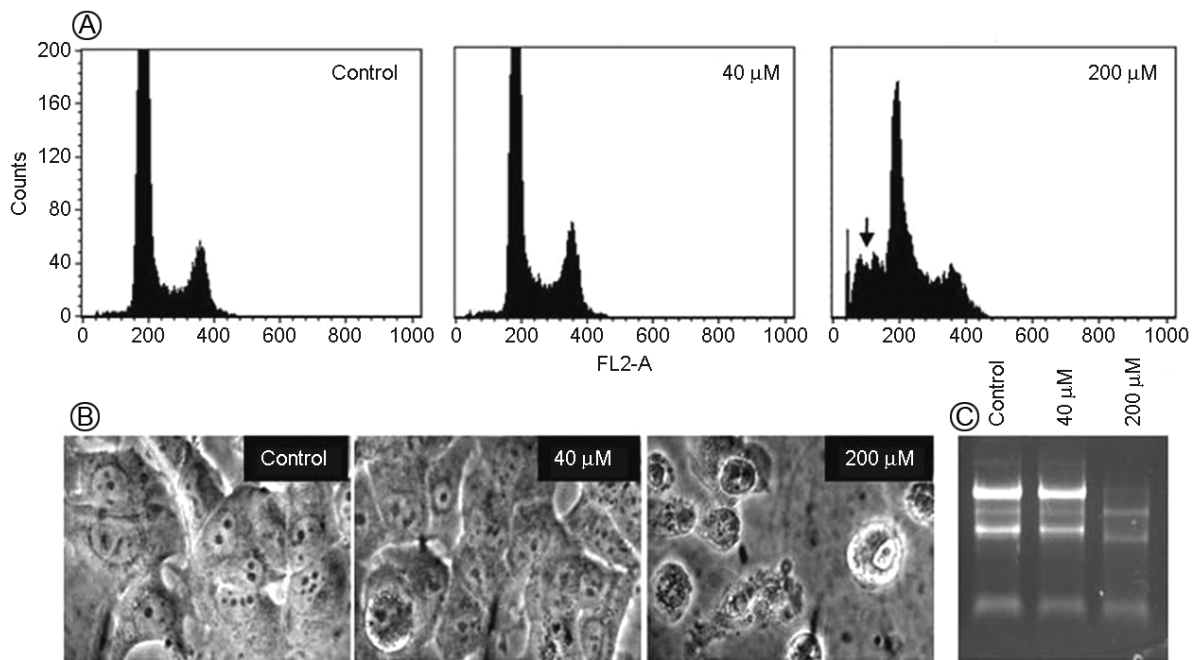

Fig. 2— Effect of 24 h EGCG treatment at indicated concentrations, on flow-cytometry profiles (A), morphology (B), and total RNA (C), of MCF-7 cells. The black arrow in the right panel in A indicates cell debris.
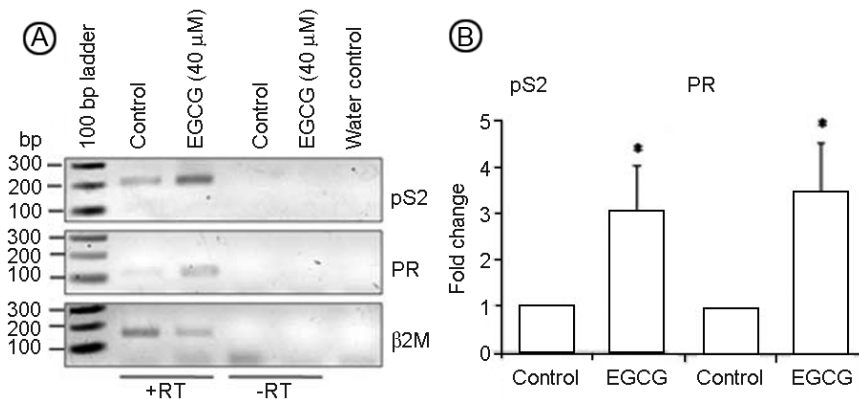

Fig. 3— Semi-quantitative RT-PCR analysis of the effect of 40 µM EGCG treatment on the steady state mRNA expression levels of pS2 and PR in MCF-7 cells. (A) Representative data from three biological replicate experiments, (B) Graphical representation of pS2 and PR mRNA expression levels in EGCG treated cells relative to control. Bars represent mean ± SD (n=3, * $P < 0.05$) of fold change in mRNA levels.

In this model, EGCG and TSA were together shown to increase the expression of PR mRNA. Increased green tea consumption has also been observed to be associated with increased expression of PR in postmenopausal breast cancer patients[22].

Taken together, it can be concluded that EGCG may exert its effect on breast cancer cells by influencing the expression of estrogen regulated genes. This needs to be addressed in greater detail by studying the effect of EGCG treatment on the complete repertoire of estrogen regulated genes using microarray technology.

Acknowledgement

References

1 Chen D, Daniel KG, Kuhn DJ, Kazi A, Bhuiyan M, Li L, Wang Z, Wan SB, Lam WH, Chan TH & Dou QP, Green tea and tea polyphenols in cancer prevention, *Front Biosci*, 9 (2004) 2618.

2 Shukla Y, Tea and cancer chemoprevention: a comprehensive review, *Asian Pac J Cancer Prev*, 8 (2007) 155.

3 Shirakami Y, Shimizu M & Moriwaki H, Cancer chemoprevention with green tea catechins: from bench to bed, *Curr Drug Targets*, 13 (2012) 1842.

4 Seely D, Mills EJ, Wu P, Verma S & Guyatt GH, The effects of green tea consumption on incidence of breast cancer and recurrence of breast cancer: a systematic review and meta-analysis, *Integr Cancer Ther*, 4 (2005) 144.

5 Sun CL, Yuan JM, Koh WP &Yu MC, Green tea, black tea and breast cancer risk: a meta-analysis of epidemiological studies, *Carcinogenesis*, 27 (2006) 1310.

6 Ogunleye AA, Xue F & Michels KB, Green tea consumption and breast cancer risk or recurrence: a meta-analysis, *Breast Cancer Res Treat*, 119 (2010) 477.

7 Baliga MS, Meleth S & Katiyar SK, Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells *in vitro* and *in vivo* systems, *Clin Cancer Res*, 11 (2005) 1918.

8 Thangapazham RL, Singh AK, Sharma A, Warren J, Gaddipati JP & Maheshwari RK, Green tea polyphenols and its constituent epigallocatechingallate inhibits proliferation of human breast cancer cells *in vitro* and *in vivo*, *Cancer Lett*, 245 (2007) 232.

9 Kushima Y, Iida K, Nagaoka Y, Kawaratani Y, Shirahama T, Sakaguchi M, Baba K, Hara Y & Uesato S, Inhibitory effect of (-)-epigallocatechin and (-)-epigallocatechingallate against heregulinbeta1-induced migration/invasion of the MCF-7 breast carcinoma cell line, *Biol Pharm Bull*, 32 (2009) 899.

10 Tran PL, Kim SA, Choi HS, Yoon JH & Ahn SG, Epigallocatechin-3-gallate suppresses the expression of HSP70 and HSP90 and exhibits anti-tumor activity *in vitro* and *in vivo*, *BMC Cancer*, 10 (2010) 276.

11 Deguchi H, Fujii T, Nakagawa S, Koga T & Shirouzu K, Analysis of cell growth inhibitory effects of catechin through MAPK in human breast cancer cell line T47D, *Int J Oncol*, 21 (2002) 1301.

12 Zhang Y, Han G, Fan B, Zhou Y, Zhou X, Wei L & Zhang J, Green tea (-)-epigallocatechin-3-gallate down-regulates VASP expression and inhibits breast cancer cell migration and invasion by attenuating Rac1 activity, *Eur J Pharmacol*, 606 (2009) 172.

13 Sen T, Dutta A & Chatterjee A, Epigallocatechin-3-gallate (EGCG) downregulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NF kappa B and AP-1 in the human breast cancer cell line MDA-MB-231, *Anticancer Drugs*, 21 (2010) 632.

14 Banerjee S, Sengupta K, Saxena NK, Dhar K & Banerjee SK, Epidermal growth factor induces WISP-2/CCN5 expression in estrogen receptor-alpha-positive breast tumor cells through multiple molecular cross-talks, *Mol Cancer Res*, 3 (2005) 151.

15 Bieche I, Parfait B, Laurendeau I, Girault I, Vidaud M, & Lidereau R, Quantification of estrogen receptor alpha and beta expression in sporadic breast cancer, *Oncogene* 20 (2001) 8109.

16 Rasband WS, Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012

17 Goodin MG, Fertuck KC, Zacharewski TR & Rosengren RJ, Estrogen receptor-mediatedactions of polyphenoliccatechins *in vivo* and *in vitro*, *Toxicol Sci*, 69 (2002) 354.

18 Kuruto-Niwa R, Inoue S, Ogawa S, Muramatsu M & Nozawa R, Effects of tea catechins on the ERE-regulated estrogenic activity, *J Agric Food Chem*, 48 (2000) 6355.

19 Farabegoli F, Barbi C, Lambertini E & Piva R, (-)-Epigallocatechin-3-gallate downregulates estrogen receptor alpha function in MCF-7 breast carcinoma cells, *Cancer Detect Prev*, 31 (2007) 499.

20 De Amicis F, Russo A, Avena P, Santoro M, Vivacqua A, Bonofiglio D, Mauro L, Aquila S, Tramontano D, Fuqua SA & Andò S, *In vitro* mechanism for downregulation of ER-α expression by epigallocatechingallate in ER+/PR+ human breast cancer cells, *Mol Nutr Food Res*, 57 (2013) 840.

21 Li Y, Yuan YY, Meeran SM & Tollefsbol TO, Synergistic epigenetic reactivation of estrogen receptor-α (ERα) by combined green tea polyphenol and histone deacetylase inhibitor in ERα-negative breast cancer cells, *Mol Cancer*, 9 (2010) 274.

22 Nakachi K, Suemasu K, Suga K, Takeo T, Imai K & Higashi Y, Influence of drinking green tea on breast cancer malignancy among Japanese patients, *Jpn J Cancer Res*, 89 (1998) 254.