Journal of Cellular Biochemistry 82:387–398 (2001)

Green Tea Extracts Decrease Carcinogen-Induced Mammary Tumor Burden in Rats and Rate of Breast Cancer Cell Proliferation in Culture

Kathryn T. Kavanagh,[1,2] Laurie J. Hafer,[1,2] Dong W. Kim,[1,3] Koren K. Mann,[2,4] David H. Sherr,[2,4] Adrianne E. Rogers,[1,2] and Gail E. Sonenshein[2,3]*

[1]Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts 02118
[2]Program in Research on Women's Health, Boston University School of Medicine, Boston, Massachusetts 02118
[3]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
[4]Department of Environmental Health, Boston University School of Public Health, Boston, Massachusetts 02118

Abstract Epidemiological evidence suggests tea (*Camellia sinensis* L.) has chemopreventive effects against various tumors. Green tea contains many polyphenols, including epigallocatechin-3 gallate (EGCG), which possess anti-oxidant qualities. Reduction of chemically induced mammary gland carcinogenesis by green tea in a carcinogen-induced rat model has been suggested previously, but the results reported were not statistically significant. Here we have tested the effects of green tea on mammary tumorigenesis using the 7,12-dimethylbenz(*a*)anthracene (DMBA) Sprague-Dawley (S-D) rat model. We report that green tea significantly increased mean latency to first tumor, and reduced tumor burden and number of invasive tumors per tumor-bearing animal; although, it did not affect tumor number in the female rats. Furthermore, we show that proliferation and/or viability of cultured Hs578T and MDA-MB-231 estrogen receptor-negative breast cancer cell lines was reduced by EGCG treatment. Similar negative effects on proliferation were observed with the DMBA-transformed D3-1 cell line. Growth inhibition of Hs578T cells correlated with induction of p27^{Kip1} cyclin-dependent kinase inhibitor (CKI) expression. Hs578T cells expressing elevated levels of p27^{Kip1} protein due to stable ectopic expression displayed increased G1 arrest. Thus, green tea had significant chemopreventive effects on carcinogen-induced mammary tumorigenesis in female S-D rats. In culture, inhibition of human breast cancer cell proliferation by EGCG was mediated in part via induction of the p27^{Kip1} CKI. J. Cell. Biochem. 82: 387–398, 2001.
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Key words: green tea; breast cancer; EGCG; DMBA; p27^{Kip1}

The incidence of breast cancer, the second leading cause of death from cancer among women in the United States, has been increasing since the 1980s. Interestingly, breast cancer mortality rates are race-dependent. In 1999, the mortality rate of breast cancer per 100,000 Caucasians was 26.0, of African–Americans 31.5, and of Asian–Americans 11.6. Studies show that migration of young Asian women to the United States dramatically increases their risk of breast cancer and mortality from breast cancer [Haenszel and Kurihara, 1968; Kelsey and Gannon, 1990; Ziegler et al., 1993]. In an effort to explain this phenomenon, epidemiologists have put forth various hypotheses, including differences in diet and environmental

Abbreviations used: GTP, green tea polyphenol; EGCG, epigallocatechin-3 gallate; DMBA, 7,12-dimethylbenz(*a*) anthracene; S-D, Sprague-Dawley; FBS, fetal bovine serum; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; ER, estrogen receptor; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; IPTG, isopropyl-beta-D-thiogalactopyranoside.

Kathryn T. Kavanagh and Dong W. Kim were the recipients of the Karin Grunebaum Cancer Research Fellowship.

Grant sponsor: Department of Army; Grant numbers: DAMD17-94-J-4468, DAMD17-98-1, DAMD17-98-1-8034, DAMD17-99-1-9083, DAMD17-94-J-442194; Grant sponsor: N.I.H.; Grant number: CA82742.

*Correspondence to: Gail E. Sonenshein, Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118. E-mail: gsonensh@bu.edu

Received 9 January 2001; Accepted 23 February 2001

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exposure to carcinogens [Haenszel and Kurihara, 1968; Kelsey and Gannon, 1990; Ziegler et al., 1993]. Dietary comparisons of the Asian diet and the typical Western diet show, among many differences, that the Asian population consumes more green tea.

Tea (*Camellia sinensis* L.) is prepared as green, black, or oolong tea. Green tea, which is made by steaming or drying fresh tea leaves at elevated temperatures, retains its chemical composition of polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids. Polyphenols make up ~40% of the dry weight of green tea leaves. The green tea polyphenols (GTPs), which include epicatechin, epicatechingallate, epigallocatechin, and epigallocatechin-3 gallate (EGCG), possess antioxidant qualities. Statistics indicate that the incidence of breast cancer in regions where green tea is consumed in large quantities, including China and Japan, is much lower than in western societies. Epidemiologic studies have indicated that green tea reduces the risk of many other cancers, including stomach, lung, colon, rectum, liver, and pancreas [Mukhtar et al., 1994; Goldbohm et al., 1996; National Cancer Institute, 1996; Yang et al., 1996; Zheng et al., 1996; Ji et al., 1997]. Concentrated, purified polyphenol extracts of green tea have been shown in laboratory animals to have anticarcinogenic activity against tumors of the duodenum [Fujita et al., 1989], esophagus [Wang et al., 1994; Stoner and Mukhtar, 1995], lung [Taniguchi et al., 1992; Wang et al., 1992a], and skin [Wang et al., 1989, 1992b; Katiyar et al., 2000]. Reduction of chemically induced mammary gland carcinogenesis by green tea has been suggested by Hirose et al. [1997] and Tanaka et al. [1997], but the results reported were not statistically significant. Further more, green tea was administered in the feed rather than the drinking fluid. In a series of three bioassays, a significant inhibitory effect of black tea on mammary tumorigenesis was found in rats fed a high fat diet [Rogers et al., 1998]. The toxicity of tea extracts is low, and they are potentially important cancer chemopreventive agents. Several possible mechanisms for the protective effects of green tea on carcinogenesis have been proposed: (1) modulation of carcinogen-metabolizing enzymes, (2) trapping of ultimate carcinogens, (3) inhibitory action against nitrosation reactions, and (4) inhibition of cell proliferation-related activities [reviewed in Stoner and Mukhtar, 1995]. Here we have examined the effects of green tea extracts on breast cancer using the Sprague-Dawley (S-D) rat carcinogen model and breast cancer cells in culture. In the rat model, ~90% of female S-D rats given a single intragastric 15–25 mg/kg dose of DMBA develop mammary tumors within 7–20 weeks [Rogers and Conner, 1990]. We find that green tea extracts given in drinking water reduce the mammary tumor burden. Second, treatment of cultured human breast cancer cells with GTPs or EGCG inhibits their growth, correlating with increased expression of the cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$. At higher doses, cell death was observed.

MATERIALS AND METHODS

Rat Studies

Virgin female S-D rats were treated according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. Forty S-D, 4-week-old female weanling rats (weighing approximately 40–50 g), were purchased from Charles River Laboratories (Wilmington, MA). They were immediately weighed and housed individually in an environmentally controlled animal facility at the Boston University Laboratory Animal Science Center. Rats were randomized into four groups of approximately equal average body weight and fed the AIN-76A purified diet. Body weights were recorded weekly. The composition of the treatment groups was as follows:

Group	No. of rats	Fluid source		
		DMBA	Water	Green tea
CG1	5	0	+	0
TG1	5	0	0	+
CG2	15	+	+	0
TG2	15	+	0	+

Green tea powder (kindly supplied by Lipton Tea Co., Inc., Englewood Cliffs, NJ) was reconstituted in deionized water to a concentration of 0.3%. (The certificate of analysis of the green tea powder indicated that the catechin composition, which had been assessed by HPLC, was as follows: 11.79% EGCG, 7.87% (−)-epigallocatechin, 5.76% (−)-epicatechin gallate, 4.21% (−)-epicatechin, 1.39% (+)-gallocatechin, 1.33% (+)-catechin, and 0.39% (+)-gallocatechin gallate). Rats in the control groups (CG1 and

CG2) were given deionized water to drink, and rats in the TG1 and TG2 groups were given 0.3% green tea as their sole fluid source beginning two days after entry into the facility. Rats were given 15 mg/kg DMBA in 0.2 ml sesame oil or 0.2 ml sesame oil alone by gastric gavage at 8 weeks of age and palpated for tumors weekly starting 4 weeks later. Rats were euthanized by CO_2 inhalation and necropsied when they bore a tumor that was $\geq$3 cm or ulcerated or at termination of the experiment at 17 weeks post-DMBA administration. All tumors, grossly normal mammary glands and uteri were excised. Sections of tumors and grossly normal glands were fixed in ice-cold 4% paraformaldehyde (4°C) for histology and immunohistochemistry. The remaining tissue was frozen on dry ice for molecular/biochemical studies. Frozen tissues were stored at −80°C.

Analysis of Tumor Endpoints

Chi-square analysis was used to compare the tumor incidences between treatment groups. One-way analysis of variance was used to compare the tumorigenesis endpoints (tumor number, weight, burden, and latency) between treatment groups. The post hoc tests used were Scheffe's and Tukey's; these were performed using SAS 6.12 and SPSS 7.0. A P-value of $\leq$0.05 was considered statistically significant.

Cell Growth and Treatment Conditions

The estrogen receptor (ER)-negative Hs578T human breast cancer cell line was derived from a mammary carcinosarcoma and is epithelial in origin [Hackett et al., 1997]. Hs578T cells were propagated in DMEM with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY), 4.5 g/L glucose, 10 μg/ml of insulin (Sigma, St. Louis, MO), and 100 μg/ml streptomycin (Gibco), and 100 U/ml penicillin (Gibco). The ER-negative MDA-MB-231 human cancer cell line was derived from a patient with poorly differentiated adenocarcinoma of the breast. The cells were grown in DMEM essentially as above except with addition of 0.29 mg/L L-glutamine. The D3-1 line was derived by DMBA-mediated transformation of ER-negative MCF-10F human mammary epithelial cells, which were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype [Soule et al., 1990]. D3-1 cells were cultured as published previously [Calaf and Russo, 1993]. GTPs and EGCG were purchased from LKT Laboratories Inc., St. Paul, MN. GTP was dissolved in ddH_2O. EGCG was dissolved in sterile 50% DMSO at a concentration of 100 mg/ml, and diluted with ddH_2O to a 1 mg/ml working strength solution. Cells were incubated with EGCG for 24, 48, or 72 h as indicated or the volume of diluted DMSO equivalent to the highest dose employed.

For the non-radioactive cell proliferation assay (Promega, Madison, WI), cells were seeded at the indicated confluence in 96-well tissue culture dishes. GTPs or EGCG was added at the indicated concentration and cultures incubated, in triplicate, for 4–6 h in the presence of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) solution (333 μg/ml) and 25 μM phenazine methosulfate according to the manufacturer's directions (Promega). The A490 was measured using an ELISA plate reader. For assessment of apoptosis, the TUNEL assay was performed using the ApopTag peroxidase kit from Intergen Company according to the manufacturer's directions. Statistical analysis was performed between the control and treated cultures at the latest time points analyzed in each experiment using the Student's t-test. A P-value $\leq$0.05 was considered statistically significant.

Immunoblot Analysis

Cells were scraped in PBS and lysed in RIPA buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS and 1% sodium sarcosyl, 0.2 mM PMSF, 10 μg/ml leupeptin, 1 mM dithiothreitol). Following incubation on ice for 5 min, the DNA was sheared either by sonication for 5–10 sec or by passing the lysate 20 times through a 23 g and then a 25 g needle. The debris was removed by centrifugation at 13,000 rpm for 30 min at 4°C and the total cellular proteins, contained in the supernatant, collected. Protein concentrations were determined using the Bio-Rad *Dc* protein assay. For immunoblot analysis, proteins were resolved on a 10% polyacrylamide-SDS gel and subjected to immunoblotting as we have described previously [Sovak et al., 1997]. The antibody against the CKI $p27^{Kip1}$ (13231A) was purchased from Pharmingen (San Diego, CA). Densitometry was performed and values presented normalized to the β-actin loading control.

Isolation of Hs578T Stable Transfectants

The LacSwitch-inducible system consists of a p3′SS eukaryotic Lac-repressor-expressing vector carrying the *lac I* gene, and a eukaryotic lac-operator-containing p27^{Kip1} vector driven by the RSV-LTR promoter, termed pOPRSVI-*p27*, prepared as described previously [Wu et al., 1999]. Hs578T cells were transfected sequentially, essentially as described previously [Sovak et al., 1997]. Briefly, the p3′SS vector was introduced by the calcium phosphate, and transfectants selected with 50 μg/ml of Hygromycin B. A mixed population of these cells (Hs578T-*lacR*) was obtained and expression of the Lac I repressor protein confirmed (data not shown). These cells were subjected to a second round of transfection with either 20 μg of pOPRSVI-*sense-p27* vector (carrying the *neo* resistance gene), or 2.5 μg of pSV2*neo* vector DNA. Double-transfected cells were selected with both hygromycin and 1,200 μg/ml G418. A mixed population of cells (Hs578T-*p27S* and Hs578T-*neo*) was isolated initially. Three single cell clones were isolated by the method of limiting dilution. For induction, cells were treated with the indicated dose of isopropyl-beta-D-thiogalactopyranoside (IPTG). The Lac I repressor protein antibody was obtained from Stratagene (#217449).

FACS Analysis

Cells were washed in cold PBS, trypsinized, and collected by centrifugation. Cells were resuspended in 0.5 ml hypotonic fluorochrome solution containing 5.0 μg/ml propidium iodide (PI, Sigma), 1% sodium citrate, and 0.1% Triton X-100 (Sigma), and analyzed in a Becton-Dickson FACScan flow cytometer, as we have described previously [Wu et al., 1996].

RESULTS

Effect of Green Tea on Mammary Tumorigenesis

In S-D rats, a single intragastric dose of DMBA induces mammary tumors in ~90% of animals within 7–20 weeks [Rogers and Conner, 1990]. To test the ability of green tea to reduce or ablate mammary tumor formation, animals were treated with 15 mg/kg DMBA, and given 0.3% green tea or water as their sole fluid source. The effects of green tea on cumulative probability of bearing a palpable mammary tumor, tumor number, burden, and latency were all assessed. No effect of tea on body weight gain was observed (data not shown). What appeared to be a slight initial reduction in probability of palpable tumors in the green tea-fed rats compared to the water-fed rats failed to reach statistical significance (Fig. 1). In tumor-bearing rats, the mean latency to first tumor was significantly longer in the green tea-fed group (84 days) than in the water-fed group (66 days) ($P = 0.001$) (Table I). Importantly, there was a statistically significant decrease in mean tumor burden per tumor-bearing animal (TBA) in the green tea-fed rats (2.5±4.5 g) compared to the water-fed rats (8.3±6.9 g) ($P = 0.03$) (Table I). The water-fed rats had 3.0 tumors/TBA, and the green tea-fed rats had 1.9; this apparent decrease was not significant (Table I). Of all tumors detected, 89% (42/47) were malignant. They were generally well-differentiated papillary adenocarcinomas; no association of histopathologic subtypes, such as comedo carcinoma, with treatment was discerned. There was no significant difference in malignant tumors/TBA with green tea consumption (1.7±1.5) compared to water-fed controls (2.8±1.7). A significant reduction was observed in the mean number of invasive malignant tumors/TBA with green tea consumption (0.2±0.4) compared to water-fed rats (1.5±1.8) ($P = 0.02$) (Table II). Green tea had no significant effect on the number of non-invasive tumors/TBA (Table II). In summary, green tea significantly increased mean latency to first

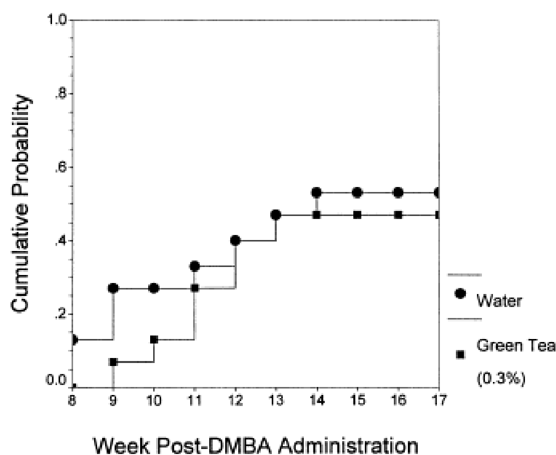

Fig. 1. Cumulative probability of bearing a palpable tumor in DMBA-treated female S-D rats as a function of green tea. Rats were given 15 mg/kg body weight DMBA at 8 weeks of age.

TABLE I. DMBA-Induced Mammary Tumor Incidence, Number, and Weight in Female S-D Rats Drinking Tea or Water

Group	Tumor incidence (%)[a]	Tumor no./TBA[b]	Tumor wt. (g)/TBA	Latency (days) to first tumor
Water (CG2)	53	3.0±1.6	8.3±6.9	66±14[d]
Green tea (TG2)	80	1.9±1.6	2.5+4.5[c]	84±21

[a]Fifteen rats per group.
[b]TBA: tumor-bearing animal; values given are mean ± SD.
[c]Significantly less than water-fed (CG2) control animals ($P = 0.03$).
[d]Significantly shorter than green tea-fed (TG2) animals ($P = 0.001$).

tumor and reduced tumor burden and number of invasive tumors/tumor-bearing animal.

GTPs Decrease Growth of Hs578T Breast Cancer Cells

In the clinic, ER-negative tumors are not responsive to anti-estrogenic treatments such as tamoxifen, and generally indicate a poorer prognosis. Therefore, we sought to determine whether treatment with green tea extracts decreases the growth of ER-negative breast cancer cells, and first selected the Hs578T cell line. Hs578T cells were treated with concentrations of GTPs ranging from 0 to 160 μg/ml. Cell growth was assessed every 24 h for a 72 h period by cell numbers using an MTS assay (Fig. 2A). Incubation in the presence of 40 μg/ml GTPs had no detectable effect on Hs578T cell growth ($P = 0.95$), whereas, 80 μg/ml GTP slowed the growth of these breast cancer cells ($P < 0.05$). No growth, i.e., no increase in cell numbers, was seen with a higher dose of 160 μg/ml GTP ($P < 0.01$). Similar effects of GTPs were obtained by cell counting using a hemocytometer (data not shown). Thus, treatment with GTPs inhibits growth of Hs578T cells in a dose-dependent fashion.

We next assessed the effects of EGCG, one of the more potent anti-carcinogenic components of GTPs, on growth of Hs578T cells (Fig. 2B). EGCG slowed the growth of the Hs578T cells in a dose-dependent fashion ($P < 0.01$ at all doses tested). A slower growth was seen at an EGCG concentration of 40 μg/ml, while no increase in cell numbers was seen at 80 μg/ml EGCG. At 160 μg/ml EGCG, a decline in cell numbers was observed (Fig. 2B). Thus, both GTP and EGCG

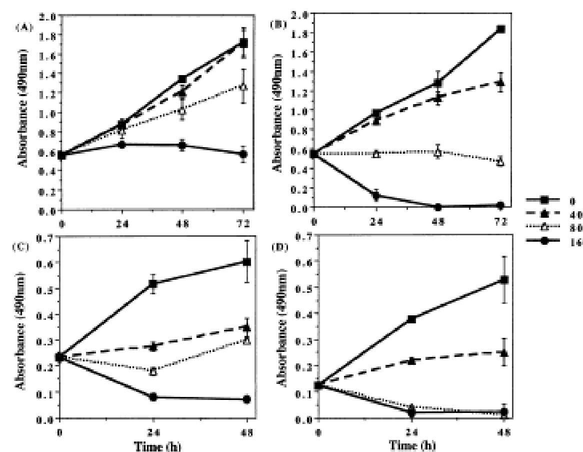

Fig. 2. GTPs and EGCG slow growth of ER-negative breast cancer cells in a dose-dependent fashion: **(A)** Effects of GTPs on Hs578T cells. Hs578T cells were plated, in triplicate, at a density of 3.9×10^3 cells/cm^2 in 96-well plates. After overnight incubation, GTP was added at a concentration of 0, 40, 80, or 160 μg/ml, as indicated. Cultures were incubated for an additional 24, 48, or 72 h. Cell proliferation was quantified by conversion of MTS dye to its formazan product read at OD490 nm. The data are presented as the mean±SD. **(B)** Effects of EGCG on Hs578T cells. Hs578T cells were plated, in triplicate, at a density of 3.9×10^3 cells/cm^2. After overnight incubation, EGCG was added at the indicated concentration dissolved in DMSO or the DMSO vehicle alone was added as control (0 μg/ml). Cultures were then incubated and processed as above in (A). **(C)** Effects of EGCG on MDA-MB-231 cells. MDA-MB-231 cells were plated, in triplicate, at a density of 6×10^3 cells/cm^2. After overnight incubation, EGCG was added and cultures processed as above. A significant decrease in cell numbers was seen at all doses ($P < 0.05$). **(D)** Effects of EGCG on D3-1 cells. D3-1 cells were plated, in triplicate, at a density of 7×10^3 cells/cm^2, and treated with EGCG and processed as above. A statistically significant decrease was achieved at 80 and 160 μg/ml EGCG ($P < 0.05$).

TABLE II. Number of Non-Invasive and Invasive Tumors per Tumor-Bearing Animal (TBA)

Histology	Group	Number/TBA[a]
Non-invasive	Water (CG2)	1.2±1.0
	Green tea (TG2)	1.5±1.4
Invasive	Water (CG2)	1.5±1.8
	Green tea (TG2)	0.2±0.4[b]

[a]Mean ± SD.
[b]Significantly less than water-fed (CG2) control animals ($P = 0.025$).

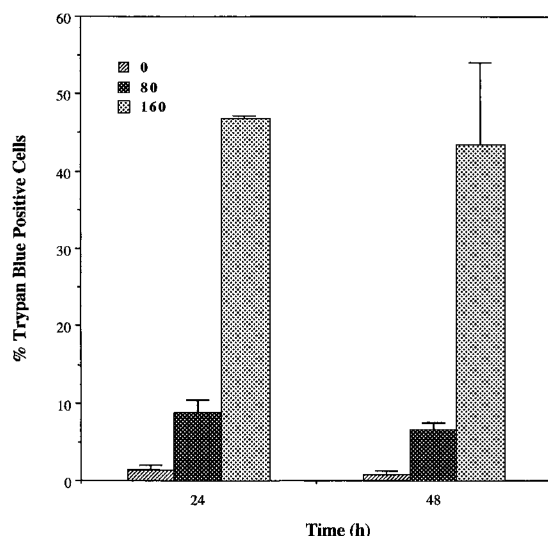

Fig. 3. Higher doses of EGCG kills Hs578T cells as measured by trypan blue staining. Hs578T cells were plated, in triplicate, at 2.6×10^3 cells/cm^2 in 6-well plates. After overnight incubation, EGCG was added at 80 or 160 μg/ml in DMSO or the DMSO vehicle alone was added as control (0 μg/ml). After an additional 24 or 48 h period, both adherent and floating cells were harvested and subjected to trypan blue analysis for dead cells. Values of the percentage of trypan blue positive cells are given as the mean±SD.

slow proliferation of Hs578T breast cancer cells. A lower concentration of EGCG (80 μg/ml) than GTP mixture (160 μg/ml) was effective in preventing an increase in cell numbers over time.

The drop in Hs578T cell numbers with the highest dose of EGCG suggested cell death was occurring. To monitor for cell death, trypan blue exclusion analysis was performed (Fig. 3). To ensure that floating, dead cells were not lost during the preparation, cells on the dish and in the media were combined and trypan blue positive and negative cells counted. At 80 μg/ml EGCG, a low percentage of the Hs578T cells stained trypan blue positive at 24 and 48 h (between 7 and 8%), indicating that the cells were largely viable. A substantial increase in the number of dead cells was seen with 160 μg/ml EGCG (~45% trypan blue positive cells) (Fig. 3). A TUNEL assay indicated apoptosis was occurring at doses of 80 and 160 μg/ml EGCG (data not shown). Taken together, these findings indicate that growth of Hs578T cells begins to slow at 40 μg/ml EGCG, while higher doses of 80–160 μg/ml EGCG inhibit growth and/or cause apoptosis of Hs578T breast cancer cells.

EGCG Slows Growth of Multiple ER-Negative Breast Cancer Cell Lines

To determine whether the growth inhibitory effects of EGCG could be extended to other ER-negative cells, the MDA-MB-231 and D3-1 breast cancer cell lines were selected. The D3-1 line was derived by DMBA-mediated transformation of non-malignant MCF-10F human mammary epithelial cells [Calaf and Russo, 1993]. MDA-MB-231 cells displayed an EGCG dose–response curve similar to that of Hs578T cells (Fig. 2C), while the D3-1 cells were somewhat more sensitive (Fig. 2D). The apparent higher sensitivity of the D3-1 line was extended to trypan blue analysis in order to assess EGCG-mediated death of D3-1 cells. A significant level of cell death was detected even at 80 μg/ml, and 90–100% of the cultured cells had died within 48–72 h of incubation in the presence of 160 μg/ml EGCG (Fig. 4). Thus, EGCG slows growth and induces death of ER-negative breast cancer cell lines in a dose-dependent fashion.

p27^{Kip1} Cyclin-Dependent Kinase Inhibitor Expression is Induced by EGCG Treatment

Since the p27^{Kip1} CKI has been implicated in control of cell cycle progression, we measured the effects of EGCG treatment on the levels of

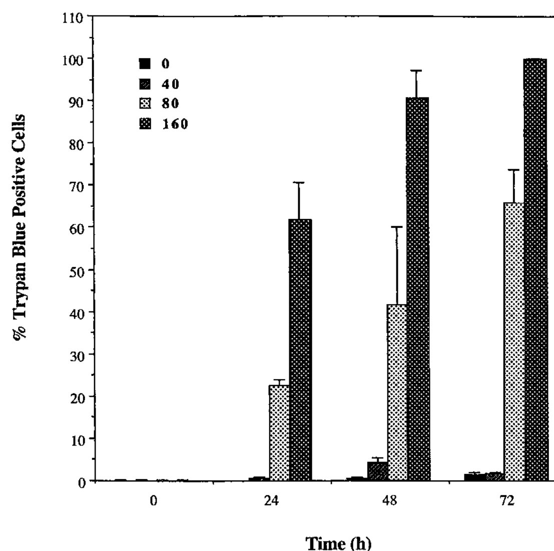

Fig. 4. EGCG reduces viability of D3-1 cells. D3-1 cells were plated, in triplicate, at 7×10^3 cells/cm^2. After overnight incubation, EGCG was added at 40, 80, or 160 μg/ml in DMSO or the DMSO vehicle alone was added as control (0 μg/ml). After an additional 24, 48, or 72 h period, both adherent and floating cells were harvested and subjected to trypan blue analysis for dead cells. Values of the percentage of trypan blue positive cells are given as the mean±SD.

$p27^{Kip1}$ CKI protein in Hs578T cells. Cultures of exponentially growing cells were treated for 24 h with 40 or 80 μg/ml EGCG, as appropriate to inhibit cell proliferation. Whole cell extracts were prepared and subjected to immunoblot analysis for $p27^{Kip1}$ protein expression (Fig. 5A). A small increase in the $p27^{Kip1}$ levels was observed in response to treatment with 40 μg/ml EGCG (1.5-fold relative to untreated control cells), which causes a slight decrease in growth. A larger increase in $p27^{Kip1}$ level was noted with 80 μg/ml EGCG (2.8-fold relative to untreated cells). We then assessed the effects of a longer (48 h) period of EGCG treatment (Fig. 5B). Growth for 48 h alone resulted in an increase in $p27^{Kip1}$ levels, likely due to the increase in cell density (2.8-fold). EGCG treatment clearly elevated the $p27^{Kip1}$ levels even further (5.5-fold). Thus, growth arrest of HS578T cells in response to EGCG treatment is accompanied by an increase in the level of expression of the CKI $p27^{Kip1}$.

Ectopic Expression of $p27^{Kip1}$ Arrests Growth of Hs578T Cells

To determine the role of induction of $p27^{Kip1}$ protein in Hs578T breast cancer cells, the

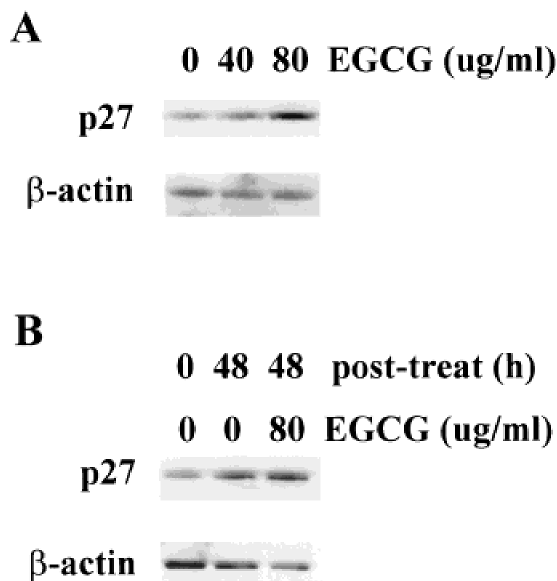

Fig. 5. EGCG induces $p27^{Kip1}$ expression: **(A)** Hs578T cells were incubated in the presence of 0, 40, or 80 μg/ml in DMSO for a 24 h period and total protein extracts prepared and subjected to immunoblotting for expression of $p27^{Kip1}$ and β-actin protein. **(B)** Twenty-four hours after plating, Hs578T cultures were either harvested directly (0 h) or incubated for 48 h in the presence of 80 μg/ml EGCG or the equivalent volume of DMSO. Total protein extracts were prepared, and analyzed as in (A).

LacSwitch inducible expression system was used. Mixed populations of stable Hs578T-*p27S* and Hs578T-*neo* population of cells were isolated. Since we have found that the response of the mixed populations is relatively minimal due to the presence of cells expressing only low levels, three single cell clones of Hs578T-*p27S* cells were isolated by limiting dilution (Hs578T-*p27S* #2, #3, and #4). To test for induction of $p27^{Kip1}$ expression, the mixed populations of Hs578T-*p27S* and Hs578T-*neo* cells, and the three Hs578T-*p27S* clones were treated with 0, 10, or 20 mM ITPG, as indicated (Fig. 6A). Total cellular proteins were extracted and samples (50 μg) subjected to immunoblot analysis for $p27^{Kip1}$ expression. A very high level of induction of the $p27^{Kip1}$ protein was detected in the Hs578T-*p27S* cells at both concentrations of IPTG (Fig. 6A, upper panel). In contrast, control Hs578T-*neo* cells showed essentially no induction of the $p27^{Kip1}$ protein. As expected, the Lac I repressor protein was detected at high levels in extracts from both cell populations, confirming equal loading (Fig. 6A, lower panel). One of these clones (Hs578T-*p27S* #3) showed a very high level of $p27^{Kip1}$ induction upon 10 mM IPTG treatment (Fig. 6B, upper panel); whereas, the two other clonal isolates (Hs578T-*p27S* #4 and Hs578T-*p27S* #2) displayed no significant increase in $p27^{Kip1}$ levels (Fig. 6B, lower panel and data not shown). Thus, we selected the Hs578T-*p27S* mixed population and Hs578T-*p27S* #3 for study of the effects of $p27^{Kip1}$ induction on cell cycle, while the Hs578T-*neo* mixed population and Hs578T-*p27S* #4 cells served as negative controls.

The Hs578T-*p27S*, Hs578T-*neo*, Hs578T-*p27S* #3, or Hs578T-*p27S* #4 cells were treated, in triplicate, with IPTG for 24 h and analyzed on a Becton-Dickson FACScan flow cytometer (Fig. 7 and Table III). Approximately, 64.4±0.4% of the Hs578T-*p27S* cells treated with IPTG were in the G0/G1 state, compared to 57.2±1.9% when untreated. As expected, the Hs578T-*neo* cells had essentially equivalent cell cycle distribution in the presence or absence of IPTG. IPTG treatment of Hs578T-*p27S* #3 cells resulted in G0/G1 arrest of 82±0.5%, compared to 62.5±1.9% in untreated cells (Fig. 7). Clone Hs578T-*p27S* #4 cells, which showed no induction of $p27^{Kip1}$ levels, displayed no significant difference in the cell cycle distribution with respect to IPTG treatment (Fig. 7). Taken together these results indicate the induction of

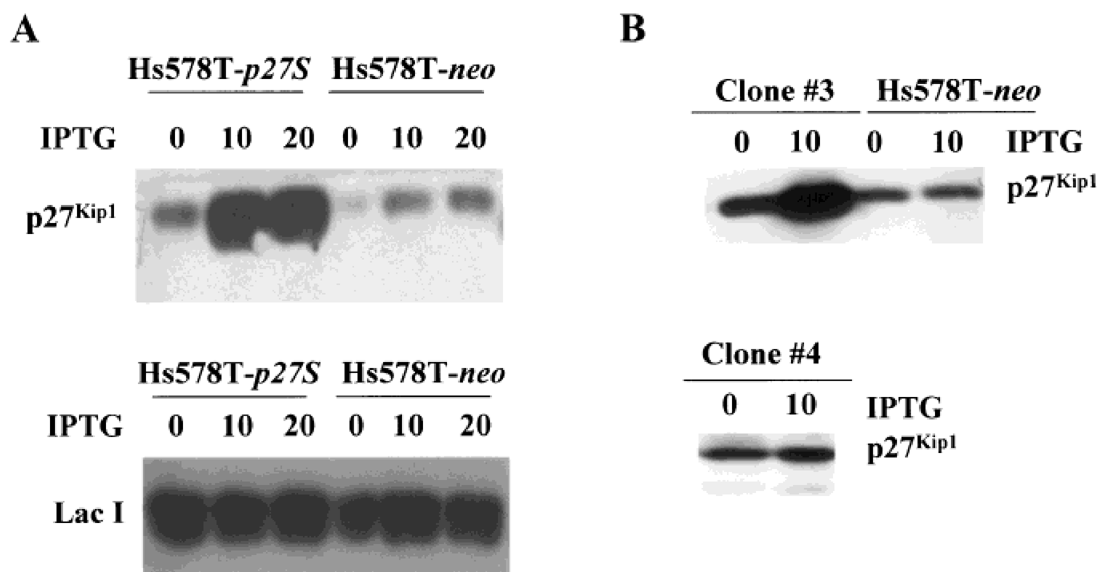

Fig. 6. Ectopic expression of $p27^{Kip1}$ protein is induced by IPTG treatment in Hs578T-*p27S* cells: **(A)** Cultures of mixed populations of Hs578T-*p27* and -*neo* cells were treated with 0, 10, or 20 mM IPTG for 28 h and total cellular proteins were extracted. Samples (50 μg) were subjected to immunoblot analysis using antibodies to the $p27^{Kip1}$ (Pharmingen 13231A) (upper panel) and the Lac I repressor protein (Stratagene #217449) (lower panel). **(B)** Three clonal isolates were derived from the Hs578T-*p27S* cells by limiting dilution, tested for induction of $p27^{Kip1}$ protein upon treatment in the absence or presence of 10 mM IPTG, as above in (A). Hs578T-*neo* cells were tested similarly, as negative control.

the $p27^{Kip1}$ protein participates in the G0/G1 arrest of Hs578T breast cancer cells.

DISCUSSION

Here we show that green tea extracts given to S-D rats in their drinking fluid significantly decrease carcinogen-induced tumor burden and invasiveness and significantly increase latency to first tumor. The cumulative probability of bearing a palpable tumor also was numerically decreased, although this was not statistically significant. Similarly, doses of GTP and EGCG between ∼40 and 80 μg/ml slowed growth of various ER-negative breast cancer cell lines in culture, including Hs578T, MDA-MB-231, and D3-1. Slower growth could be related in part to induction of $p27^{Kip1}$ protein, which causes an arrest of cells at the G1/S phase transition. Higher doses of EGCG (∼80 to 160 μg/ml) were shown to cause death of breast cancer cells, at least in part due to apoptosis as judged by TUNEL assay (data not shown). Thus, these data extend the findings of other groups on the anti-carcinogenic properties of green tea [reviewed in Yang et al., 1997]. Overall, these

TABLE III. Summary of FACS Analysis of Hs578T-*p27S* and Hs589T-*neo* Cells

	Cell types	Average G0/G1±SD
Hs578T-*p27S*	MP untreated	57.2±1.9
	MP + IPTG	64.4±0.4
	Clone #3 untreated	62.5±1.9
	Clone #3 + IPTG	82.1±0.5
	Clone #4 + untreated	60.6±1.0
	Clone #4 + IPTG	60.6±1.6
Hs578T-*neo*	MP untreated	56.0±1.1
	MP + IPTG	58.4±1.1

FACS analysis was performed to determine the cell cycle distribution of the cells upon induction of $p27^{Kip1}$ protein in the mixed population (MP) of Hs578T-*p27S* cells, and the clonal populations (Clones #3 and 4). The MPs Hs578-*p27S*, Clones #3 and 4, and MP Hs578-*neo* control cells were treated in triplicate with 20 mM IPTG. After 24 h, the cells were washed in cold PBS, trypsinized and pelleted, and resuspended in 0.5 ml hypotonic flurochrome solution containing 5.0 μg/ml PI, 1% sodium citrate, and 0.1% Triton X-100. Cells were then analyzed in Becton-Dickson FACScan flow cytometer for cell cycle distribution. The results of the average cell cycle distribution of the cells are summarized above.

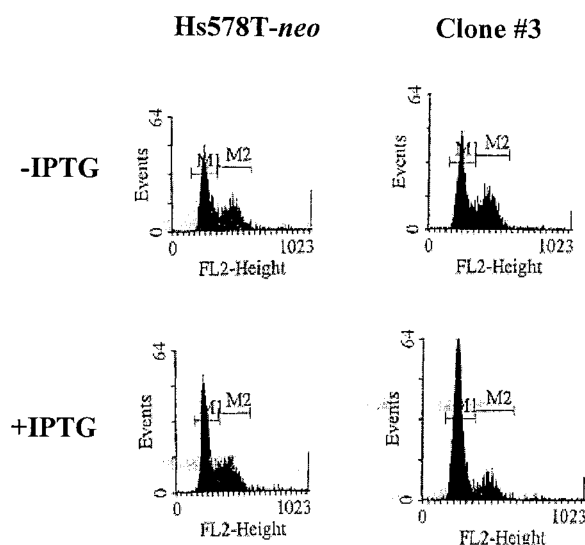

Fig. 7. FACS analysis of individual Hs578T-*p27S* clones. Hs578T-*p27S* #3 clonal populations and Hs578T-*neo* cells were treated in triplicate with 10 mM IPTG for 24 h, and analyzed on a Becton-Dickson FACScan flow cytometer for cell cycle distribution. The upper panel shows representative flow cytometry results for Hs578T-*p27S* #3 and the Hs578T-*neo* cells in the absence or presence of IPTG.

results confirm the anti-carcinogenic properties of GTPs on mammary tumor formation in vivo. Importantly, the data presented here were obtained, for the first time, using drinking fluid as the route of administration of the green tea, rather than feed, thus modeling human exposure to GTPs. We demonstrate also the ability of GTPs to slow growth of breast cancer cells in culture. These observations provide both in vivo and cell culture evidence in support of a role for green tea components in chemoprevention and, possibly, chemotherapy of breast cancer.

The $p27^{Kip1}$ CKI was originally characterized as a protein able to interrupt CDK2 kinase activity in TGF-β1-treated cells, and in a yeast two hybrid system as being able to interact with cyclin D and CDK4 [Polyak et al., 1994; Toyoshima and Hunter, 1994]. CKI $p27^{Kip1}$ is now known to bind to and inhibit complexes formed by cyclin E-CDK2, cyclin A-CDK2, and cyclin D-CDK4, playing essential roles in transition through the G1 phase, in particular the restriction point [Sherr and Roberts, 1999]. Here, we demonstrate that increased levels of $p27^{Kip1}$ can prevent cell cycle progression of Hs578T breast cancer cells, with arrest occurring prior to entry into S phase. The ability of EGCG to induce $p27^{Kip1}$ expression in breast cancer cells provides a mechanism for the observed reduction in growth rate. Similar effects on $p27^{Kip1}$ were reported recently by Liang et al. [1999]. Interestingly, a high level of ectopic expression of $p27^{Kip1}$ failed to result in apoptosis or cell death of Hs578T cells, indicating that other molecular events are induced by EGCG treatment.

Interestingly, many human malignancies, including cancers of the breast, stomach, colon, lung, esophagus, prostate, and pituitary [reviewed in Cariou et al., 1998; Lloyd et al., 1999], are typified by very low basal levels of $p27^{Kip1}$ protein. Reduced $p27^{Kip1}$ protein levels has been correlated with more aggressive, poorly differentiated cancers [Fredersdorf et al., 1997; Cariou et al., 1998; Han et al., 1999] and indicate a poorer prognosis [Catzavelos et al., 1997; Gillett et al., 1999; Han et al., 1999]. In breast cancer, several studies have correlated decreased $p27^{Kip1}$ levels with increased malignant behavior. Catzavelos et al. [1997] completed an informative study of 168 breast cancer patients, who had undergone primary surgery between 1986 and 1992. Patients were divided into two categories based on their degree of elevation of $p27^{Kip1}$ protein levels. High $p27^{Kip1}$ protein levels were found in patients with less aggressive types of tumor, while lower levels were detected in those with a high grade of malignant tumor. Furthermore, those patients displaying axillary lymph node involvement displayed similar or even lower $p27^{Kip1}$ protein levels in the nodes in question. Moreover, Kaplan–Meier curves of disease-free survival were constructed and indicated a significantly shorter disease-free survival with decreased $p27^{Kip1}$ protein levels. These findings led the investigators to conclude that a decrease in $p27^{Kip1}$ level facilitates breast cancer progression. Similar results were obtained from a study of 102 female breast cancer patients with lymph node metastasis conducted by Tsuchiya et al. [1999]. In this study, a significant positive correlation was also found between $p27^{Kip1}$ levels and ER status. Similarly, Gillett et al. [1999] observed that high $p27^{Kip1}$ protein levels were associated with smaller, well-differentiated, more slowly proliferating tumors with ER-positive status. In addition, these patients were also shown to have a significantly longer survival than those with low $p27^{Kip1}$ levels, unless they also had high levels of cyclin D1. In effect, overexpression of cyclin D1 appeared to overcome the detrimental effect of low $p27^{Kip1}$

levels. Similar results have been shown by other groups [Porter et al., 1997; Tan et al., 1997].

In animal models, green tea extracts inhibit chemical carcinogenesis, including cancers of the gastrointestinal tract, lung, and skin in mice [Fujita et al., 1989; Wang et al., 1989, 1991, 1992a,b, 1994, 1995; Conney et al., 1992; Katiyar et al., 1992; Taniguchi et al., 1992; Pingzhang et al., 1994]. It has been reported also that EGCG inhibits the growth of human breast and prostate tumors transplanted into athymic mice [Liao et al., 1995]. Two studies of mammary gland carcinogenesis in rats provided suggestive evidence of a chemopreventive effect of green tea [Hirose et al., 1994, 1995]. One study of rats fed a high fat diet and given black tea to drink showed a reduction in tumor multiplicity compared to rats given water [Weisburger et al., 1997]; results in rats fed control diet were not reported. Another study using a diet containing 1% green tea catechins fed to female S-D rats showed that tea was effective in reducing mammary gland tumorigenesis in the promotion, but not the progression, stages of carcinogenesis [Hirose et al., 1997]. Consistent with these findings, several studies have also found that green tea treatment of breast cancer cells in vitro reduces their rate of proliferation [Araki et al., 1995; Chen et al., 1998; Liang et al., 1999]. In these studies, we have shown the effects of green tea on carcinogen-induced tumor burden in rats and extended the in vitro findings to additional cell lines.

ACKNOWLEDGMENTS

We thank J. Russo for generously providing cell lines and Lipton Tea company for providing green tea. We thank G. Zanieski for assistance in preparation of the Hs578T transfectants, J. Foster for the use of the scanning densitometer, and D. Sloneker for assistance in preparation of this manuscript.

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