

Green Tea Inhibits Vascular Endothelial Growth Factor (VEGF) Induction in Human Breast Cancer Cells

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ABSTRACT Investigators have shown that green tea and its main catechin epigallocatechin-3 gallate (EGCG) may decrease the risk of cancer. Our previous study showed that green tea extract (GTE) as well as its individual catechin components inhibited MDA-MB231 breast cancer cell and human umbilical vein endothelial cell (HUVEC) proliferation. Further, GTE suppressed breast cancer xenograft size and decreased the tumor vessel density in vivo. In the current study, we investigated the effect of GTE on the major angiogenic factor vascular endothelial growth factor (VEGF) in an in vitro experiment. GTE or EGCG (40 mg/L) significantly decreased the levels of the VEGF peptide secreted into conditioned media. This occurred in both HUVEC and human breast cancer cells and the effect was dose dependent. Furthermore, GTE and EGCG decreased the RNA levels of VEGF in MDA-MB231 cells. This inhibition occurred at the transcriptional regulation level and was accompanied by a significant decrease in VEGF promoter activity. We also showed that GTE decreased c-fos and c-jun RNA transcripts, suggesting that activator protein (AP)-1-responsive regions present in the human VEGF promoter may be involved in the inhibitory effect of GTE. Furthermore, GTE suppressed the expression of protein kinase C, another VEGF transcription modulator, in breast cancer cells. Inhibition of VEGF transcription appeared to be one of the molecular mechanism(s) involved in the antiangiogenic effects of green tea, which may contribute to its potential use for breast cancer treatment and/or prevention. *J. Nutr.* 132: 2307–2311, 2002.

KEY WORDS: • green tea • vascular endothelial growth factor • epigallocatechin-3 gallate • breast cancer • angiogenesis.

Many epidemiologic studies have shown that green tea may decrease the risk of cancer (1). A distinction should be made between green tea and black tea. Most reports showing positive cancer preventive effects are from studies of Asians who drink predominantly green tea (2), whereas studies of Europeans who drink black tea only infrequently found protective effects (3). The cancer preventive effect of green tea is observed in pancreatic, colon and rectal cancers (4). Some other studies support this phenomenon in colorectal cancer but others do not (5–7). Stomach cancer has been studied with mixed results (8), but it seems that high consumption of tea at moderate temperature is beneficial (9–12). Green tea has been observed to decrease the risk of esophageal cancer (13). The effect on lung cancer is not definitive (14,15). Finally, there are reports linking the consumption of green tea with an improved prognosis in breast cancer (16–18).

The mechanisms of the cancer preventive effect of green tea are being explored. Recently, we examined the effect of green tea on the growth of breast cancer and endothelial cells

in both in vitro assays and animal models. We demonstrated that both green tea extract (GTE)⁴ as well as its individual catechin components were effective in inhibiting breast cancer and endothelial cell proliferation. In mouse experiments, GTE suppressed xenograft size and decreased the tumor vessel density (19). In the present study, we investigated the effects of GTE and epigallocatechin-3-gallate (EGCG) on angiogenic factor vascular endothelial growth factor (VEGF) expression with in vitro studies.

MATERIALS AND METHODS

Reagents. GTE was obtained from Pharmanex batch #990222 (Brisbane, CA). GTE was characterized by HPLC, and its catechin components were described recently (19). EGCG and phorbol myristate acetate (PMA) were purchased from Sigma Chemical (St. Louis, MO).

Cell culture. Human umbilical vein endothelial cell (HUVEC) was purchased from Cascade Biologics (Portland, OR). The cells were plated on tissue culture flasks coated with 1.5% gelatin (Difco,

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⁴ Abbreviations used: AP-1, activator protein 1; DMEM, Dulbecco's minimum essential medium; EGCG, epigallocatechin-3 gallate; FCS, fetal calf serum; GTE, green tea extract; HUVEC, human umbilical vein endothelial cell; PKC, protein kinase C; PMA, phorbol myristate acetate; VEGF, vascular endothelial growth factor.

Detroit, MI) in PBS. They were maintained in endothelial growth media containing basic fibroblast growth factor, heparin, fetal calf serum (FCS), penicillin, streptomycin, and amphotericin-B (Cascade Biologics, Portland, OR). The human breast cancer cell line MDA-MB231 was obtained from American Type Culture Collection (Rockville, MD) and maintained on nongelatinized flasks in Dulbecco's minimum essential medium (DMEM; Life Technologies, Grand Island, NY) with 10% heat-inactivated FCS, 100,000 U/L penicillin, and 100 mg/L streptomycin.

Quantification of VEGF peptide levels. The conditioned media were prepared with confluent cultures of either HUVEC or MDA-MB231 cells. Briefly, the serum-free DMEM media bathing the cells over various time durations of treatment were collected, centrifuged at $250 \times g$ to remove debris and frozen until further analysis. Cell lysates were prepared for both VEGF level and total protein concentration determinations. Total protein was estimated by the Bradford method using a colorimetric assay (Bio-Rad, Hercules, CA). VEGF concentrations were determined as described by the manufacturer using a quantitative ELISA (R&D Systems, Minneapolis, MN). All analyses and calibrations were carried out in duplicate. The amount of VEGF immunoreactivity was calculated using recombinant human VEGF standards present on each microtiter plate. Optical densities were determined at 450 nm using a microtiter plate spectrophotometer (LabSystems, Helsinki, Finland). The blank reading was subtracted from the duplicate values for both standards and samples. A standard curve was created using StatView 5.01 (SAS Institute, Cary, NC) by plotting the logarithm of the mean absorbance of each standard vs. the logarithm of the VEGF concentration.

RNA extraction and Northern analysis. Total RNA was extracted from cells using the Trizol Reagent (GIBCO BRL Life Technologies, Grand Island, NY). Northern blot hybridization was performed. Briefly, total RNA (25 μ g) was subjected to electrophoresis on 1% denaturing formaldehyde-agarose gels, transferred to a Hybond-N+ positively charged nylon membrane (Amersham, Piscataway, NJ) overnight by capillary elution, and UV cross-linked at 120,000 μ J/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The blots were prehybridized for 6–8 h at 42°C in rapid hybridization buffer (Amersham), and the membranes were then hybridized overnight at 42°C with a human VEGF-specific cDNA probe (20), and *c-fos* or *c-jun* cDNA probes (21). Probes were purified by agarose gel electrophoresis using the QIAEX Gel Extraction kit (Qiagen, Chatsworth, CA). Each cDNA probe was radiolabeled with (α -³²P) deoxyribonucleotide triphosphate using the random-priming technique with the Rediprime labeling system (Amersham). The probed nylon membranes were then washed and exposed to radiographic film. All blots were also reprobed for β -actin (Clontech, Palo Alto, CA) content to verify RNA quantity. Bands for Northern blots were quantitated using a Molecular Dynamic Laser Densitometer (Model PSD1) and an Image Quant Version 0.1 software program (Frederick, MD).

VEGF promoter reporter activity. The effect of green tea extract on the transcriptional regulation of VEGF in MDA-MB231 cells was examined using transient transfection with a VEGF promoter (luciferase)-reporter construct. Full-length VEGF promoter cDNA was subcloned into pGL3 by standard techniques (22). The following plasmids were used: pGL3-VEGF (containing the human VEGF promoter linked to the firefly luciferase reporter gene) (Promega, Madison, WI), pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to a constitutively active *Renilla* luciferase reporter gene) and pGL3 (plasmid vector alone as a negative control). MDA-MB231 cells (1.0×10^6) were seeded into 6-well plates, and the pRLTK and pGL3-VEGF constructs were cotransfected into cells with the FuGENETM6 Transfection Reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. pRLTK and pGL3 were cotransfected as negative controls. After cells were incubated in the transfection media for 24 h, the media were changed to serum-free media. To determine whether green tea could inhibit the increase in VEGF-promoter activity associated with serum deprivation, cells were treated with GTE or EGCG. After a 48-h incubation, cells were harvested with passive lysis buffer (Dual-Luciferase Reporter Assay

System, Promega), and luciferase activity was determined with a single sample luminometer, as outlined in the manufacturer's protocol.

Western analysis. To evaluate protein kinase C (PKC) protein presence, cells were pelleted after appropriate treatments. Pellets were lysed in 0.05 mol/L Tris (pH 7.4), 0.15 mol/L NaCl, and 1% Nonidet P-40, then sonicated for 10 s to ensure complete lysis. The cell lysate was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad). Cell protein (50 μ g) was separated by a 10% Tris-HCl Ready Gel (Bio-Rad) and transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked overnight (4°C) with 10 g/L bovine serum albumin, and 10% FCS in PBS-0.3% Tween 20, and then incubated with a 1:200 dilution of anti-PKC Monoclonal Antibody (LC Laboratories, Woburn, MA) for 2 h. The blots were then washed six times with PBS-Tween 20, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. The blots were then washed four times with PBS-Tween 20. Immunoreactive bands were developed with a chemiluminescent substrate (Super Signal, Pierce, IL), and visualized by autoradiography (Hyperfilm ECL, Amersham).

Statistical analysis. Means and SEM were used to summarize the VEGF measurement for each cell line with different culture conditions or VEGF promoter activity. Two-way ANOVA was used to test whether concentration, length of culture or their interaction affected the VEGF values. Pair-wise comparisons for different doses and culture times were also performed using pair-wise *t*-test. $P < 0.05$ was used as the statistical significance level for all tests.

RESULTS

Effect of GTE or EGCG on VEGF secretion into conditioned media of human breast cancer cells and HUVEC. The control and GTE- and EGCG-treated MDA-MB231 human breast cancer cells (Fig. 1A) and HUVEC (Fig. 1B) differed ($P = 0.006$). The GTE- and EGCG-treated cells did not differ and there were no dose by time interactions.

In MDA-MB231 human breast cancer cells (Fig. 2A), GTE had significant dose ($P = 0.0001$) and time ($P < 0.0007$) effects on VEGF peptide secretion, but there was no dose by time interaction. Increasing GTE concentration decreased VEGF secretion and peptide levels increased as the culture time increased. In HUVEC (Fig. 2B), 20 and 40 mg/L GTE decreased VEGF secretion at all time points tested ($P = 0.0001$), but there was no effect of 10 mg/L GTE until 48 h incubation. The time effect was significant only between 8 and 24 h vs. 48 and 72 h ($P = 0.0169$). The dose by time interaction was not significant.

Effect of GTE and EGCG on VEGF transcripts in human breast cancer cells. We then examined the effect of GTE and EGCG on VEGF transcript levels. When the MDA-MB231 human breast cancer cells were treated in serum-free conditions, GTE inhibited VEGF induction, particularly at 48 h (Fig. 3A,B). EGCG had a similar effect at the same dosage of 40 mg/L ($P < 0.05$) (data not shown).

Effect of GTE on VEGF promoter activity in human breast cancer cells. To examine further the effect of GTE on the transcriptional regulation of VEGF, we transiently transfected promoter-reporter constructs into MDA-MB231 human breast cancer cells. Cells transfected with pGL3-VEGF (promoter-reporter construct) and pRLTK (internal control) demonstrated an increase in VEGF promoter activity secondary to serum starvation, as expected. Treatment with either 20 or 40 mg/L GTE or EGCG inhibited induction in the activity of the VEGF promoter ($P < 0.05$) (Fig. 4).

Effect of GTE on basal and PMA-stimulated *c-fos* and *c-jun* transcripts in human breast cancer cells. To evaluate the effect of GTE treatment on *c-fos* expression, MDA-MB231 human breast cancer cells were cultured for 48 h in the

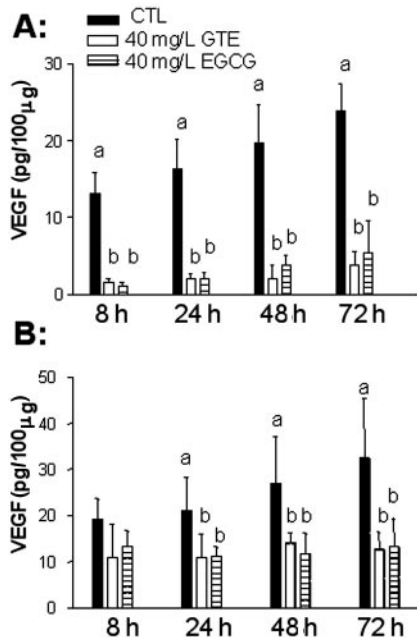


FIGURE 1 Levels of vascular endothelial growth factor (VEGF) peptide secreted into conditioned media of MDA-MB231 human breast cancer cells (A) and that of human umbilical vein endothelial cells (HUVEC, B). Cells were incubated in plain media in the absence (CTL) or presence of 40 mg/L of either green tea extract (GTE) or epigallocatechin-3-gallate (EGCG) for 8–72 h. VEGF levels were determined by ELISA, as detailed in Materials and Methods, and expressed as pg VEGF/100 μ g total cell protein content. Data are means \pm SEM of three experiments. Values in a panel that do not share a letter differ ($P < 0.05$).

absence or presence of 40 mg/L GTE. At the end of this period, cells were or were not stimulated by 50 nmol/L PMA for 30 min. GTE-treated MDA-MB231 cells had lower basal and PMA-stimulated *c-fos* RNA levels than control cells (Fig. 5). The same phenomena were observed for *c-jun* expression in these cells (Fig. 6).

Effect of GTE on PKC protein levels in human breast cancer cells. MDA-MB231 cells were cultured for 48 h with or without GTE or EGCG. As shown in Figure 7, both GTE and EGCG decreased PKC protein levels.

DISCUSSION

This study provides further evidence that green tea extract inhibits breast cancer angiogenesis. In our previous study, we demonstrated that both green tea extract as well as its individual catechin components were effective in inhibiting breast cancer and endothelial cell proliferation. In mouse experiments, green tea extract suppressed xenograft size and decreased the tumor vessel density (19). Yang et al. (23) recently reviewed the anticancer properties of green tea constituents.

Because all solid tumors are angiogenesis dependent, the suppression of endothelial cells would contribute to the overall tumor inhibition. Swiercz et al. (24) observed that EGCG reduced angiogenesis in the chicken embryo chorioallantoic membrane model. Cao and Cao (25) showed that EGCG inhibited bovine capillary endothelial cells in culture, with a 50% reduction at an EGCG concentration of 50 mg/L. In other published studies, green tea increased the frequency of apoptotic endothelial cells in a mouse model of human non-Hodgkin's lymphoma (26).

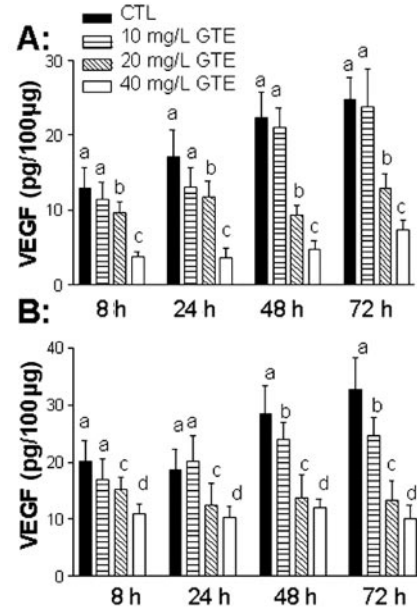


FIGURE 2 Levels of vascular endothelial growth factor (VEGF) peptide secreted into conditioned media of MDA-MB231 human breast cancer cells (A) and that of HUVEC (B). Cells were incubated in plain media in the absence (CTL) or presence of 10–40 mg/L of green tea extract (GTE) for 8–72 h. VEGF levels were determined by ELISA, as detailed in Materials and Methods, and expressed as pg VEGF/100 μ g total cell protein content. Data are means \pm SEM of three experiments. Values in a panel that do not share a letter differ ($P < 0.05$).

Angiogenesis is a complex process with many steps involving soluble factors, adhesion molecules, proteases and cytokines. Recent reports of the effect of known angiogenic growth factors on the endothelium have advanced our understanding of the mechanisms of angiogenesis at a molecular level. The best-studied angiogenic growth factor is VEGF (27). VEGF binds to the receptor VEGF-R2 (Flk1) and sends a classical

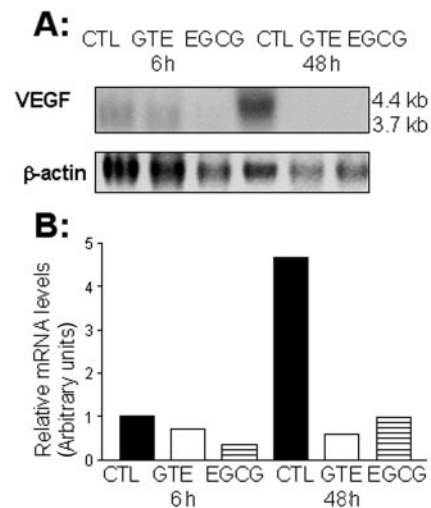


FIGURE 3 Northern analysis of vascular endothelial growth factor (VEGF) transcript in human breast cancer cells. MDA-MB231 cells were cultured in serum-free media in the absence (CTL) or presence of 40 mg/L of either green tea extract (GTE) or epigallocatechin-3-gallate (EGCG) for 6–48 h. RNA (25 μ g) was hybridized with VEGF and β -actin cDNA probes. Data are from one representative experiment of three.

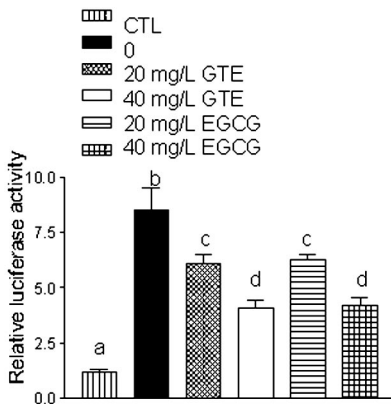


FIGURE 4 Analysis of vascular endothelial growth factor (VEGF) promoter activity in human breast cancers. MDA-MB-231 cells were cotransfected with pGL3 and pRLTK as a negative control (*lane 1*). For the positive control, cells were cotransfected with pGL3-VEGF (a VEGF promoter-luciferase-reporter construct) and pRLTK (control for transfection efficiency) (*lane 2*). After 24 h, cells were changed to serum-free media, and 20–40 mg/L of either green tea extract (GTE) or epigallocatechin-3-gallate (EGCG) was added (*lanes 3–6*). Data are means \pm SEM of three experiments. Values in a panel that do not share a letter differ ($P < 0.05$).

proliferative signal to the endothelial cell. Subsequently, VEGF binding to another receptor, VEGF-R1 (Flt1), elicits endothelial cell-cell interactions and capillary formation.

In our present study, we demonstrated that 40 mg/L GTE or EGCG greatly inhibits VEGF protein secretion in conditioned media. This inhibition occurred at the level of transcriptional regulation, which was manifested by a decrease in transcript levels and a decrease in VEGF promoter activity. This phenomenon was recently observed in colon cancer cells (22). The VEGF promoter region contains several potential binding

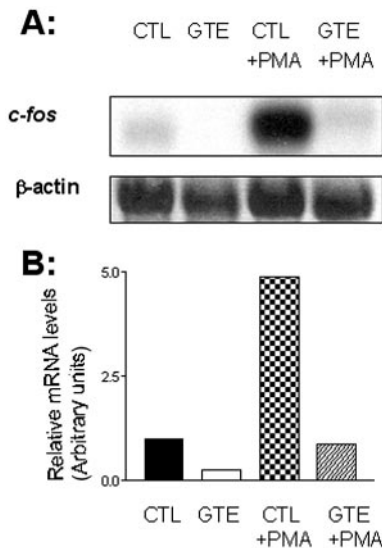


FIGURE 5 Northern analysis of *c-fos* transcript in human breast cancer cells. MDA-MB231 cells were cultured in serum-free media in the absence (CTL) or presence of 40 mg/L of GTE for 48 h. At the end of this period, cells were or were not stimulated with phorbol myristate acetate (PMA) for 30 min. RNA (25 μ g) was hybridized with *c-fos* and β -actin cDNA probes. Data are from one representative experiment of three.

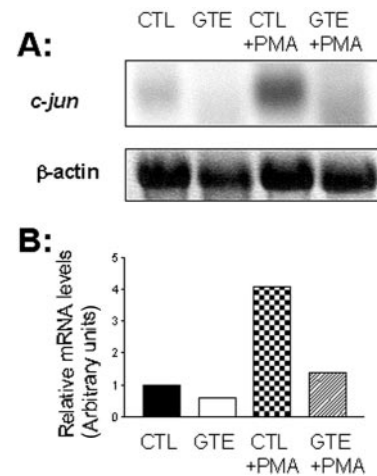


FIGURE 6 Northern analysis of *c-jun* transcript in human breast cancer cells. MDA-MB231 cells were cultured in serum-free media in the absence (CTL) or presence of 40 mg/L of green tea extract (GTE) for 48 h. At the end of this period, cells were or were not stimulated with phorbol myristate acetate (PMA) for 30 min. RNA (25 μ g) was hybridized with *c-jun* and β -actin cDNA probes. Data are from one representative experiment of three.

sites for the transcription factor activator protein-1 (AP-1) (28), and *c-fos* and *c-jun* are components of the AP-1 transcription factor complex (29). Our data showed an inhibition of *c-fos* and *c-jun* expression in both unstimulated and PMA-stimulated MDA-MB231 cells by GTE. We further explored PKC, another modulator of VEGF expression (30). PKC expression in breast cancer cells was inhibited by both GTE and EGCG. These results are consistent with reports in other cell types of PKC and AP-1 inhibition exerted by various tea components (31–33).

The inhibition of breast cancer angiogenesis by green tea likely involves multiple pathways other than VEGF transcrip-

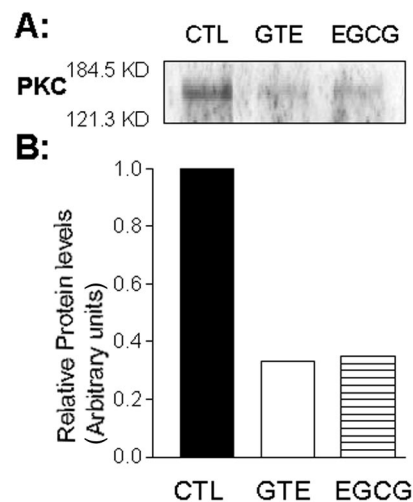


FIGURE 7 Western analysis of protein kinase C (PKC) expression in human breast cancer cells. MDA-MB231 cells were cultured in serum-free media in the absence (CTL) or presence of 40 mg/L of either green tea extract (GTE) or epigallocatechin-3-gallate (EGCG) for 48 h. Cells were lysed, and Western blot analysis was carried out as detailed in Materials and Methods, and standardized with total cell protein content. Data are from one representative experiment of three.

tion. It has been reported in other cell types that green tea inhibits other angiogenic molecules, i.e., urokinase (34), matrix metalloproteinases (MMP-2 and MMP-9) (35), and platelet-derived growth factor (36). Tumor necrosis factor- α gene expression has also been shown to be inhibited by EGCG (17). A recent abstract suggested that another mechanism involves the suppression of interleukin-8 production by endothelial cells (37).

The observation that green tea is antiangiogenic is very important clinically. Currently, there is much effort to develop antiangiogenic drugs to treat cancer. Many of these agents have completed Phase I clinical trials, and are currently in Phases II and III (38). A major shortcoming of the vast majority of the antiangiogenic drugs is that they require intravenous or subcutaneous administration. This is particularly problematic because antiangiogenic drugs have to be given on a long-term basis to control cancer growth. Furthermore, many of these compounds are complex peptides that are difficult and expensive to produce in the quantities and purities required for human use. Thus, an antiangiogenic agent that can be administered orally and inexpensively, such as green tea, would be clinically very useful. To date, a recent report of a Phase I clinical trial of green tea has been encouraging (39).

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