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Green Tea Polyphenols Modulate Secretion of Urokinase Plasminogen Activator (uPA) and Inhibit Invasive Behavior of Breast Cancer Cells

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Abstract: *Many epidemiological studies have suggested that consumption of green tea may decrease the risk of cancer. The chemopreventive effect of green tea polyphenols (GTP) has been demonstrated through the inhibition of cell proliferation and angiogenesis in cell culture and animal models of breast cancer. Metastasis of breast cancer is the major reason for the high mortality of breast cancer patients and is directly linked to the invasive behavior of breast cancer cells. Cancer metastasis consists of several interdependent processes including cancer cell adhesion, cancer cell migration, and invasion of cancer cells. In this study, we evaluated the effect of GTP on human breast cancer cells, and we show that in addition to inhibiting cell growth, GTP also suppressed the invasive behavior of MDA-MB-231 cells. These anti-invasive effects of GTP were the result of the inhibition of constitutively active transcription factors AP-1 and NF-κB, which further suppressed secretion of urokinase plasminogen activator (uPA) from breast cancer cells. Based on these results, it can be hypothesized that GTP treatment resulted in the inhibition of formation of signaling complexes responsible for cell adhesion and migration (uPA, uPA receptor, vitronectin, integrin receptor) and cell invasion (uPA, uPA receptor). Our results indicate that GTP may contribute to the anticancer effects of green tea by inhibiting the invasive behavior of cancer cells.*

Introduction

One-third of newly diagnosed cancers in women every year in the United States are breast cancers (1). Although early diagnosis and therapeutic and surgical interventions have increased the survival of breast cancer patients, breast cancer remains the leading cause of cancer death among women 20–59 yr old (1). The major reason for such a high mortality of breast cancer is the highly invasive behavior of breast cancer cells, which results in the development of cancer metastasis. Cancer metastasis results from several interdependent processes including cancer cell adhesion, cancer cell migration, and invasion of cancer cells into surrounding tissue (2). It is known that cancer metastasis is regulated, in part, by the serine protease urokinase-type plasminogen activator (uPA), which possesses proteolytic activity through the degradation of extracellular matrix (ECM) components. uPA also possesses nonproteolytic activity, which is responsible for cell adhesion and migration (3).

Some epidemiological studies have suggested that tea consumption may decrease the risk of cancer. This was confirmed by experimental studies that have demonstrated the inhibition of oral, esophagus, stomach, intestine, lung, prostate, and skin cancers in laboratory animal models (4,5). Although the inhibition of tumorigenesis was demonstrated with polyphenols from green tea and theaflavins from black tea, the cancer-preventive effects are usually linked to the consumption of green tea (6–8). However, to date, the effect of green tea on breast cancer remains inconclusive. For example, a lower risk of breast cancer in association with green-tea intake was reported (9); another study did not find any association between lower breast cancer risk and consumption of green tea (10). Nevertheless, experimental studies have demonstrated that one of the major biologically active components of green tea, (-)-epigallocatechin-3-gallate (EGCG), suppresses the growth of breast cancer cells by inhibiting the phosphatidylinositol 3-kinase, Akt kinase, and NF-κB pathways (11,12). It has also been shown that EGCG and green tea extract suppressed growth of human breast cancer xenografts in mice (13,14).

We have recently demonstrated that the transcription factors AP-1 and NF-κB are constitutively active in invasive breast cancer cells and that their inhibition results in the downregulation of expression and secretion of uPA and suppression of motility of cancer cells (15–17). In this study, we examined the effect of green tea polyphenols (GTP) on the noninvasive human mammary epithelial cells MCF-10A and

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Table 1. Polyphenol Composition of Green Tea Extract

Polyphenol	% Amount
Epigallocatechin gallate (EGCG)	48.6
Epicathechin gallate (ECG)	12.3
Epigallocatechin (EGC)	4.2
Epicatechin (EC)	4.1
Gallocatechin gallate (GCG)	1.8
Gallocatechin (GC)	1.8
Catechin	1.2
Gallic acid	0.2

highly invasive human breast cancer cells MDA-MB-231. Based on our results, we propose the molecular mechanism by which green tea may inhibit the growth and invasive potential of breast cancers.

Materials and Methods

Materials

GTP was purchased from P. L. Thomas and Co. (Morristown, NJ) as a decaffeinated powdered extract. Table 1 shows the composition of the GTP in the extract used in this study.

Cell Culture

MCF-10A human mammary epithelial cells and MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). MCF-10A cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 5% horse serum (HS), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (50 U/ml), and streptomycin (50 U/ml). MDA-MB-231 cells were maintained in DMEM containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Media and supplements came from GIBCO BRL (Grand Island, NY). HS and FBS were obtained from Hyclone (Logan, UT).

Cell Proliferation Assay

Cell proliferation was determined by the tetrazolium salt method according to the manufacturer's instructions (Promega, Madison, WI). Briefly, MCF-10A and MDA-MB-231 cells were cultured in a 96-well plate and treated at indicated times with GTP (0–100 μg/ml). At the end of the incubation period, the absorption was determined with a plate reader at 570 nm as described (18). Data points represent mean ± SD in one experiment repeated at least twice.

Cell Cycle Analysis

MDA-MB-231 cells (0.75×10^6) were seeded and after 24 h treated with GTP (100 μg/ml) for the indicated period of time (0–48 h). After incubation, the cells were harvested by trypsinization, washed with Dulbecco's phosphate-buffered saline, and resuspended in propidium iodine (50 μg/ml). Cell cycle analysis was performed on a FACStar^{PLUS} flow cytometer (Becton-Dickinson, San Jose, CA) as previously described (19). Data are the mean ± SD from 3 independent experiments.

Cell Adhesion, Migration, and Invasion Assays

Cell adhesion was performed with Cytomatrix Adhesion Strips coated with human vitronectin (Chemicon International, Temecula, CA). Briefly, MDA-MB-231 cells were treated with GTP (0–100 μg/ml) for 24 h, harvested, and counted. Cell adhesion was determined after 1.5 h of incubation at 37°C (20). Cell migration of MDA-MB-231 cells was assessed in Transwell chambers in the DMEM containing 10% FBS (21). Invasion of MDA-MB-231 cells treated with GTP (0–100 μg/ml) was assessed in Transwell chambers coated with 100 μl of Matrigel™ (BD Biosciences, Bedford, MA) diluted 1:4 with DMEM after 72 h of incubation (21).

Anchorage-Independent Growth

MDA-MB-231 cells were harvested and seeded in 6-well plates coated with 1% agarose. Anchorage-independent growth was assessed after incubation for 10–14 days with culture media with or without GTP (0–100 μg/ml), which was replaced every 4 days. Plates were stained with 0.005% Crystal Violet, and the colonies were counted manually under a microscope and photographed (21).

Reporter Gene Assays

MDA-MB-231 cells were transfected with AP-1-CAT (15) and NF-κB-Luc (BD Biosciences Clontech, Palo Alto, CA) reporter gene constructs and control β-galactosidase expression vector pCH110, as previously described (16). Cells were treated with GTP (0–100 μg/ml) for 24 h at 37°C and cell extracts were prepared 24 h after transfection. Normalized cell extracts (β-galactosidase units) were used in liquid chloramphenicol acetyl transferase (for AP-1-CAT) and luciferase assays (for NF-κB-Luc), respectively (16,22). Data points represent the average ± SD of 3 to 6 independent transfection experiments.

uPA Secretion

DMEM media from MDA-MB-231 cells treated with GTP (0–100 μg/ml) for 24 h were collected and concentrated, and the secretion of uPA was detected by Western blot

analysis with anti-uPA antibody (Oncogene Research Products, Cambridge, MA) (16).

Statistical Analysis

Data are presented as means ± SD. Statistical comparison between the control group (0 µg/ml of GTP) and groups with different GTP doses were carried out using two-sided Student's *t*-tests. The value of $P < 0.05$ was considered to be significant. Variability assays (intra-assays and interassays) were calculated and a figure of 10% or less considered to be satisfactory.

Results

GTP Inhibits Proliferation of Breast Cancer Cells by Cell Cycle Arrest at G2/M Phase

Because green tea extract containing EGCG as a major component inhibited the growth of breast cancer cells (14), we compared the effect of GTP on nontumorigenic mammary epithelial cells and highly invasive breast cancer cells. Therefore, we treated MCF-10A and MDA-MB-231 with GTP (0–100 µg/ml) for 24 and 48 h and the determined cell proliferation. As seen in Fig. 1, 100 µg/ml of GTP significantly inhibited proliferation of MDA-MB-231 breast cancer cells after 24 or 48 h of incubation, whereas the same concentration of GTP did not affect or slightly increased proliferation of nontumorigenic MCF-10A breast cells. To investigate the mechanism by which GTP inhibits growth of breast cancer cells, we analyzed cell cycle distribution by flow cytometry. We found that treatment with GTP (100 µg/ml) caused cell cycle arrest at G2/M phase as indicated by the increased amount of MDA-MB-231 cells at G2/M from 21% (0 h) to 27% (24 and 48 h) (Table 2, Fig. 2).

GTP Inhibits Invasive Behavior of Breast Cancer Cells

Because green tea extract inhibited the growth of breast cancer xenografts (14), and GTP inhibited prostate cancer metastasis in mice (23), we evaluated the effect of GTP on the invasive behavior of breast cancer cells. As mentioned previously, the ability of cancers to invade and metastasize is characterized by increased cell adhesion, cell migration, and proteolytic activity of cancer cells. To investigate whether GTP affects the adhesion of breast cancer cells, MDA-MB-231 cells were pretreated with GTP (0–100 µg/ml) for 24 h, and cell adhesion to ECM protein vitronectin was determined. As seen in Fig. 3A, GTP markedly inhibited cell adhesion. Next, we evaluated the effect on GTP on cell migration, which is one of the characteristics of highly invasive MDA-MB-231 breast cancer cells (16). MDA-MB-231 cells were pretreated with GTP (0–100 µg/ml) for 1 h, and cell migration was determined after an additional 5 h of incubation. As seen in Fig. 3B, GTP inhibited migration of breast cancer cells in a dose-dependent manner. Finally, we investigated whether GTP inhibits the proteolytic activity of MDA-MB-231 cells by cell invasion assay. The cells were seeded on the Matrigel-coated filters in the presence of GTP (0–100 µg/ml), and the amount of invaded cells was determined. GTP markedly inhibited invasion of MDA-MB-231 cells through Matrigel (Fig. 3C), thereby confirming the potency of GTP in suppressing the proteolytic activity of highly invasive cancer cells.

The ability of cancer cells to form colonies in vitro (anchorage-independent growth) strongly correlates with

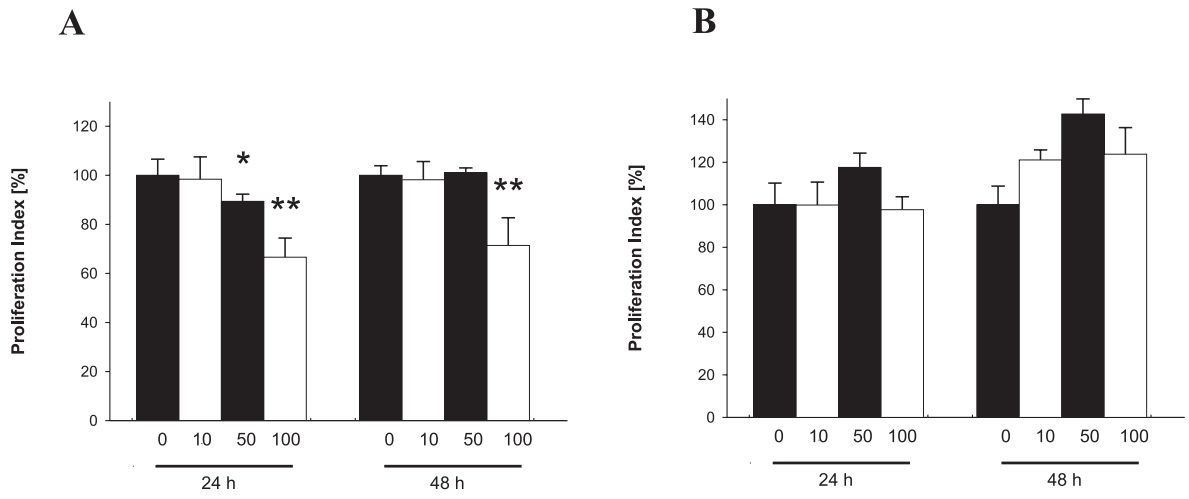


Figure 1. Green tea polyphenols (GTP) inhibits proliferation of breast cancer cells. A: MDA-MB-231 and B: MCF-10A cells were treated with 0, 10, 50, and 100 µg/ml of GTP. Proliferation was assessed after 24 and 48 h as described in the **Materials and Methods** section. Each bar represents the mean ± SD of three experiments. *$P < 0.05$, **$P < 0.01$ vs control (0 GTP).

Table 2. Effect of GTP on Cell Cycle Distribution[a,b]

Time (h)	GTP (μg/ml)	G0/G1	S	G2/M
0	0	57 ± 1.2	23 ± 0.6	21 ± 0.7
24	0	66 ± 2.1	19 ± 3.1	15 ± 1.8
48	0	75 ± 0.7	8 ± 1.9	17 ± 1.9
0	100	52 ± 1.2	27 ± 1.1	21 ± 0.4
24	100	45 ± 1.0	29 ± 2.7	27 ± 2.3*
48	100	49 ± 2.5	25 ± 3.1	27 ± 0.1**

a: Cell cycle distribution G0/G1, S, and G2/M in %. GTP, green tea polyphenols.

b: Statistical significance is as follows: *, $P < 0.05$; **, $P < 0.01$ for cells at G2/M (24 h, 48 h) vs control (0 h).

tumorigenesis in vivo (24). To determine whether GTP inhibits colony formation, we assessed the anchorage-independent growth of MDA-MB-231 cells. MDA-MB-231 cells formed colonies on 1% agarose after 14 days of incubation. However, increased concentration of GTP did not suppress colony formation of breast cancer cells (data not shown), suggesting that GTP-induced inhibition of proliferation, adhesion, migration, and invasion of breast cancer cells is probably modulated by signaling pathways distinct from pathways involved in colony formation.

GTP Inhibits Secretion of uPA From MDA-MB-231 Cells

We have previously demonstrated that inhibition of secretion of uPA is associated with the inhibition of AP-1 and NF-κB and suppresses the motility of highly invasive breast cancer cells (15,16). Therefore, we investigated whether suppression of the invasive behavior of MDA-MB-231 by GTP is mediated by the same mechanism. Because EGCG reduced AP-1-dependent transcriptional activity, AP-1-DNA binding activity (25), and NF-κB DNA binding (11), we investigated the effect of GTP on breast cancer cells that contain high levels of constitutively active AP-1 and NF-κB. MDA-MB-231 cells were transiently transfected with a reporter gene construct AP-1-CAT. The cells were treated with GTP (0–100 μg/ml) for 24 h, and the activity of AP-1 was determined. As seen in Fig. 4A, GTP markedly inhibited constitutively active AP-1 in a dose-dependent manner. To determine whether GTP also affects the activity of transcription factor NF-κB, we transfected MDA-MB-231 cells with a reporter gene construct NF-κB-Luc and treated the cells with GTP as described previously. As in the case of constitutively active AP-1, GTP inhibited constitutively active NF-κB in a dose-dependent manner (Fig. 4B). Finally, we investigated whether GTP also inhibits secretion of uPA from breast cancer cells. MDA-MB-231 cells were treated for 24 h with GTP (0–100 μg/ml), and secreted uPA was detected by Western blot analysis in collected media. In accordance with our recent report demonstrating that specific inhibitors of AP-1 and NF-κB suppressed secretion of uPA from MDA-MB-231 cells (15,16), GTP treatment also markedly suppressed the secretion of uPA from MDA-MB-231 cells in a dose-response manner (Fig. 5). The secretion of uPA was also suppressed simultaneously with the inhibition of NF-κB activity in the same cells (not shown), further confirming that GTP modulates expression and secretion of uPA in MDA-MB-231 cells.

Discussion

In this study, we evaluated the effect of GTP on the invasive behavior of breast cancer cells. Here we have shown that GTP suppressed cell growth in highly invasive MDA-MB-231 breast cancer cells, whereas the growth of

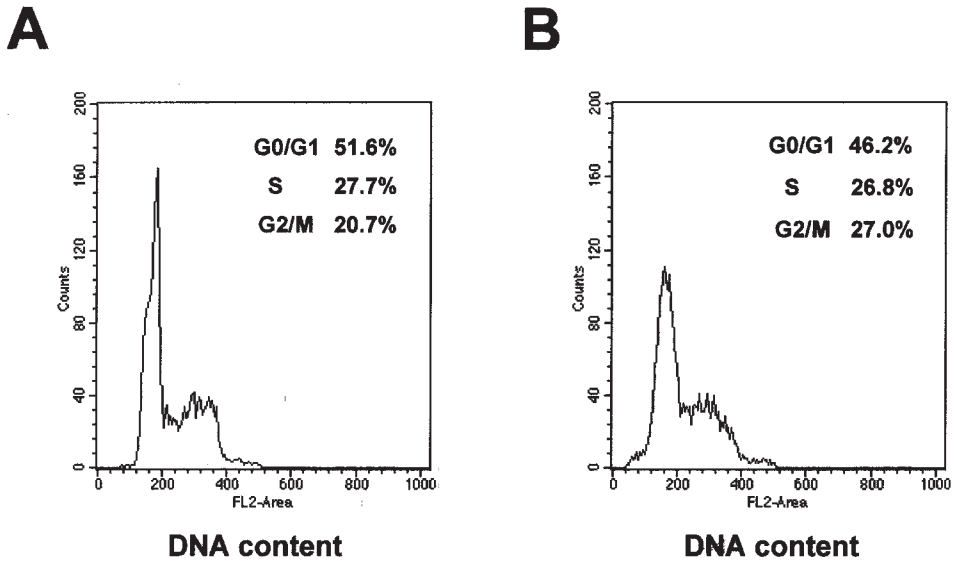


Figure 2. Green tea polyphenols (GTP) induces cell cycle arrest at G2/M phase. MDA-MB-231 cells were treated with GTP (100 μg/ml) for A: 0 h and B: 48 h, respectively. Cell cycle distribution was evaluated by flow cytometry as described in the **Materials and Methods** section. The histograms are representative of 3 independent experiments.

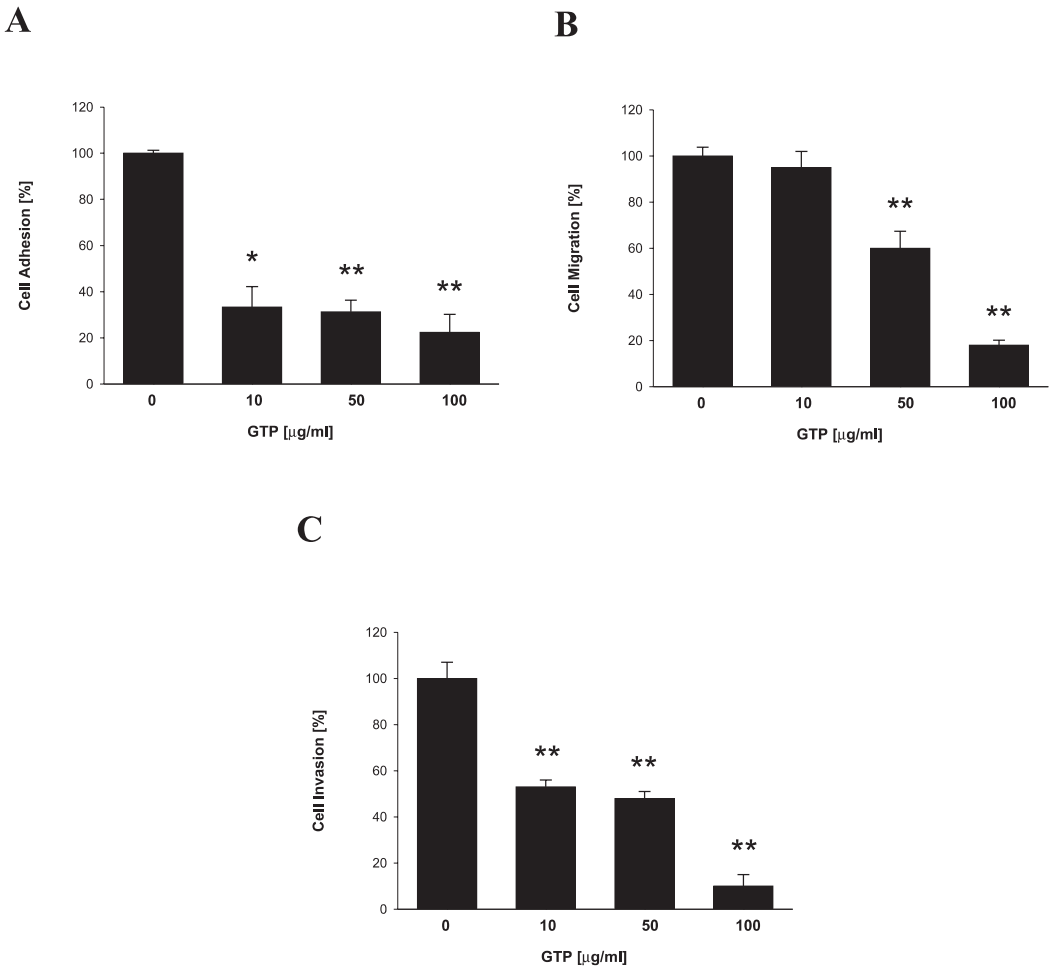
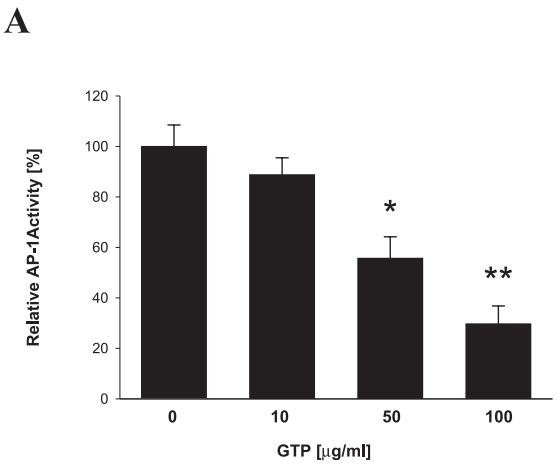


Figure 3. Green tea polyphenols (GTP) suppresses invasive behavior of MDA-MB-231 cells. A: Effect of GTP on cell adhesion. MDA-MB-231 cells were pretreated for 24 h with GTP (0–100 μg/ml), and cell adhesion to vitronectin was determined after 1.5 h of incubation as described in the **Materials and Methods** section. Data are the mean ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. B: Effect of GTP on cell migration. MDA-MB-231 cells were pretreated for 1 h with GTP (0–100 μg/ml), and cell motility was determined after 5 h of incubation as described in the **Materials and methods** section. Data are the mean ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. C: Effect of GTP on cell invasion. MDA-MB-231 cells were harvested and treated with GTP (0–100 μg/ml). Invasion through Matrigel was assessed after 72 h of incubation as described in the **Materials and Methods** section. Data are the mean ± SD of triplicate determinations. Similar results were obtained in at least two additional experiments. $*P < 0.05$, $**P < 0.01$ vs control (0 GTP).

MCF-10A mammary epithelial cells was not inhibited. Furthermore, GTP suppressed invasiveness of MDA-MB-231 cells by inhibiting cell adhesion, cell migration, and proteolytic degradation of the ECM. Our data suggest that the inhibition of breast cancer invasiveness by GTP is a result of the inhibition of AP-1 and NF-κB followed by the suppression of uPA secretion from MDA-MB-231 cells.

A large body of evidence suggests that the chemopreventive effect of GTP is caused by the modulation of specific signaling pathways in cancer cells. Here we have shown that GTP inhibited transcriptional activity of AP-1 and NF-κB in MDA-MB-231 cells at the transactivation level. Our observation is in agreement with recent reports that have demonstrated a decrease in RNA transcripts c-fos and c-jun components of the AP-1 transcription factor in breast cancer cells (26) and inhibition of NF-κB DNA binding by GTP in mice (27). Inhibition of AP-1 and NF-κB by GTP resulted in the suppression of uPA secretion from MDA-MB-231 cells, which corresponds to the downregulation of uPA expression through the inhibition of transcriptional activity of AP-1 and NF-κB, which are in the promoter region of uPA (28,29). More recently, Kim et al. (30) demonstrated that EGCG suppressed uPA expression in fibrosarcoma cells by inhibiting the uPA promoter activity and also by destabilizing uPA mRNA.

Here we have also shown that GTP suppresses crucial processes responsible for cancer metastasis: cell adhesion, cell migration, and cell invasion. Cell invasion is the result of the activity of proteolytic enzymes such as matrix metalloproteinases (MMPs), cysteine proteases, and serine proteases (31–33). Serine protease uPA binds to its receptor (uPAR) and converts plasminogen to plasmin, which degrades ECM components (34). Plasmin can further activate transforming growth factor β1 and degrade ECM indirectly through the stimulation of MMPs (34). Thus, the inhibition of uPA secretion by GTP results in the inhibition of the



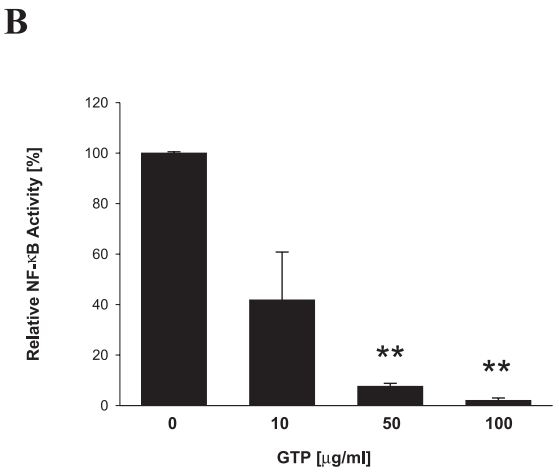


Figure 4. Green tea polyphenols (GTP) inhibits transactivation activity of AP-1 and NF-κB. A: AP-1-CAT activity after treatment with GTP. MDA-MB-231 cells were transfected with AP-1-CAT reporter gene construct and β-galactosidase expression vector pCH110. The cells were treated with GTP (0–100 μg/ml) 24h after transfection, and CAT activity was determined as described in the **Materials and Methods** section. Data are the mean ± SD of triplicate determinations. B: NF-κB-Luc activity after treatment with GTP. MDA-MB-231 cells were transfected with NF-κB-Luc reporter gene construct and β-galactosidase expression vector pCH110. The cells were treated with GTP (0–100 μg/ml) 24 h after transfection for an additional 24 h. Luciferase activity was measured as described in the **Materials and Methods** section. Data are the mean ± SD of triplicate determinations. $*P < 0.05$, $**P < 0.01$ vs control (0 GTP).

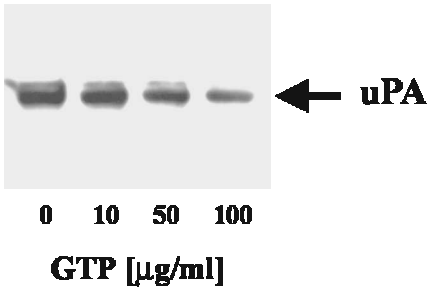


Figure 5. Green tea polyphenols (GTP) inhibits secretion of urokinase-type plasminogen activator (uPA) from MDA-MB-231 cells. Medium from MDA-MB-231 cells treated with GTP (0–100 μg/ml) for 24 h was concentrated as described in the **Materials and Methods** section, and secretion of uPA was detected by Western blot analysis with anti-uPA antibody. The results are representative of three separate experiments.

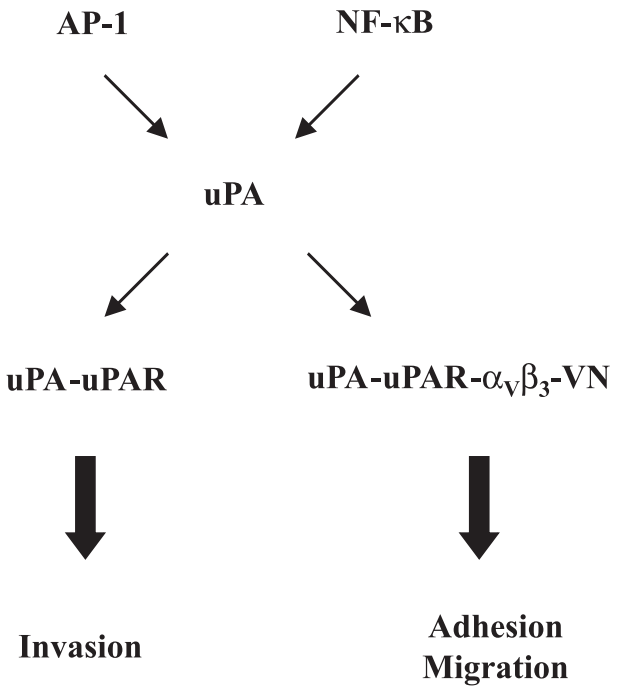


Figure 6. Scheme depicting the role of green tea polyphenols (GTP) in the inhibition of invasiveness of cancer cells. Urokinase plasminogen activator (uPA) binds to uPA receptor (uPAR), and the proteolytic activity of uPA is responsible for the degradation of ECM and cell invasion. uPA also binds to uPAR and forms a complex with $\alpha_V\beta_3$ integrin receptor, which binds vitronectin (VN), and the whole complex activates the intracellular signaling responsible for cell adhesion and migration (3). GTP inhibits transcriptional activity of AP-1 and NF-κB in highly invasive breast cancer cells, resulting in the suppression of uPA secretion and inhibition of cell adhesion, migration, and invasion.

proteolytic activity of uPA and suppression of cell invasion (Fig. 6). Inhibition by GTP of cell adhesion and cell motility is also mediated through the uPA-uPAR receptor signaling. Secreted uPA binds to uPAR and forms a complex with integrin receptor $\alpha_V\beta_3$, which is ligated to vitronectin (3). Furthermore, Wong et al. (35) demonstrated that integrin receptor $\alpha_V\beta_3$ is involved in adhesion and migration of breast cancer cells. Thus, inhibition of uPA secretion will suppress the formation of uPA-uPAR-$\alpha_V\beta_3$-vitronectin complex, which results in the inhibition of adhesion and motility of MDA-MB-231 cells (Fig. 6).

In this study, we used GTP extracts (0–100 μg/ml) containing approximately 50% of EGCG, which corresponds to 0–100 μmol/L of EGCG. This concentration is in accordance with that used in previously published studies with different cancer cell lines that have usually demonstrated the effect of EGCG at a concentration of >20 μmol/L (36). In addition,

another study demonstrated that breast cancer cells MDA-MB-231 are usually more resistant to EGCG, and the study required concentrations of 30–160 μg/ml of EGCG (12,14,37). Here we have also demonstrated that only a higher concentration of GTP (100 μg/ml) inhibits proliferation, whereas concentrations of 10 or 50 μg/ml are sufficient for the inhibition of NF-κB and AP-1 and uPA secretion, resulting in the inhibition of cell adhesion, migration, and invasion. This observation would suggest that although GTP can control invasive behavior of breast cancer cells through the modulation of NF-κB and AP-1 activities, an additional mechanism is probably required for the inhibition of proliferation of cancer cells. This mechanism could include activation of the $p27^{Kip1}$ protein (a cyclin-dependent kinase inhibitor) because only a higher concentration of EGCG (80 μg/ml) was necessary to inhibit cell proliferation by stimulating $p27^{Kip1}$ in breast cancer cells (37).

The bioavailability and tissue levels of tea constituents are key factors determining the effectiveness of tea in inhibiting tumor formation (38). A brewed cup of green tea usually contains up to 300 mg of EGCG (39), and oral administration of 20 mg green tea solid/kg of body weight in human volunteers resulted in plasma EGCG of 77.9 μg/L (40). In addition, animal studies with radioactively labeled EGCG have demonstrated 10% of the initial dose (radioactivity) in the blood and 1% in other tissues (41,42). Therefore, it is unlikely that EGCG concentration used in our experiments (5–50 μg/ml) can be obtained in breast tissue only by the regular consumption of green tea.

In summary, our data demonstrate one of the possible anticancer mechanisms of green tea by the suppression of invasiveness of breast cancer cells through the inhibition of uPA secretion. Although epidemiological studies have suggested the preventive effect of green tea against cancer, here we have shown the anticancer effect of GTP against invasive behavior of cancer cells in vitro, which corresponds to cancer metastasis in vivo. However, further studies are necessary to evaluate the effect of GTP on cancer invasion and metastasis in in vivo models.

Acknowledgments and Notes

We thank Dr Karen Spear for editing the manuscript and Colin Terry and Kathleen Bem for the statistical analysis. This work was supported by the Ross Product Division of Abbott Laboratories. Address correspondence to D. Sliva, Cancer Research Laboratory, Methodist Research Institute, 1800 North Capitol Avenue, E504, Indianapolis, IN 46202. Phone: 317–962–5731. FAX: 317–962–9369. E-mail: dsliva@clarian.org.

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