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The combination of green tea and tamoxifen is effective against breast cancer

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Epidemiologic data have suggested that green tea may prevent breast cancer. Studies in our laboratory have provided evidence that green tea extract inhibits breast cancer growth by a direct anti-proliferative effect on the tumor cells, as well as by indirect suppressive effects on the tumor-associated endothelial cells. In this study, we asked whether concurrent administration of green tea may add to the anti-tumor effects of standard breast cancer therapy. We observed that green tea increased the inhibitory effect of tamoxifen on the proliferation of the ER (estrogen receptor)-positive MCF-7, ZR75, T47D human breast cancer cells *in vitro*. This combination regimen was also more potent than either agent alone at increasing cell apoptosis. In animal experiments, mice treated with both green tea and tamoxifen had the smallest MCF-7 xenograft tumor size, and the highest levels of apoptosis in tumor tissue, as compared with either agent administered alone. Moreover, the suppression of angiogenesis *in vivo* correlated with larger areas of necrosis and lower tumor blood vessel density in treated xenografts. Green tea decreased levels of ER-α in tumors both *in vitro* and *in vivo*. We also observed that green tea blocked ER-dependent transcription, as well as estradiol-induced phosphorylation and nuclear localization of mitogen-activated protein kinase. To our knowledge, this study is the first to show the interaction of green tea with the ER pathway, as well as provide mechanistic evidence that the combination of green tea and tamoxifen is more potent than either agent alone in suppressing breast cancer growth. These results may lead to future improvements in breast cancer treatment and prevention.

Introduction

Despite multiple advances in the treatment for breast cancer, the mortality remains high (1). Since there is no definitive cure for advanced breast cancer, prevention strategies can potentially save many lives. Current effective breast cancer prevention strategies for high-risk women and for adjuvant therapy in estrogen receptor-α(ER-α) positive cancer cases include tamoxifen (2). However, a 5-year course of tamoxifen may involve serious side effects. Thus, alternative interventions such as substances from natural herbal sources are needed to replace or to supplement current regimens.

Next to water, tea is the most widely consumed beverage in the world, and its ingestion in reasonable doses is considered safe (3). There are reports linking the consumption of green tea with an improved prognosis in breast cancer (4,5). EGCG (epigallocatechin-3-gallate), the major component of green tea, was shown to suppress the growth of the breast cancer cell line Hs578t (6) and the ER-positive MCF-7 xenograft (7). Studies in our laboratory have provided evidence that green tea extract (GTE) inhibits breast cancer growth by a direct anti-proliferative effect on tumor cells, as well as by indirect suppressive effects on tumor-associated endothelial cells (8–11). Our studies characterized anti-proliferative effects of individual catechin components of green tea and determined their relative potency (8). It has been generally reported that EGCG accounts for most, if not all, of the actions of green tea. We, on the other hand, observed that CG (catechin gallate), GC (gallocatechin), EC (epicatechin), EGC (epigallocatechin), ECG (epicatechin-3-gallate) and GCG (gallocatechin gallate) suppress both ER-negative MDA-MB231 breast cancer cells and vascular endothelial cells. In fact, many of these catechin components are as potent as GTE or EGCG in our experiments. This explains why GTE has a similar potency as EGCG dose per dose, although it contains only half as much EGCG. Globally, this observation supports our philosophy that natural products exert their positive effects based on the sum or synergy of their multiple mixed components. Therefore, it may not necessarily always be beneficial to isolate one single chemical compound and concentrate it in the form of a pure drug.

The above results led to our interest in the question whether concurrent administration of green tea has any additive or synergistic effects on the widely used breast cancer prevention drug tamoxifen. Thus, we set out to determine if the combination of green tea and tamoxifen is more potent than either agent administered alone, and to elucidate any mechanistic interaction(s) between green tea, estrogen and tamoxifen. Our findings suggest that green tea may have a supplemental role, with or without tamoxifen, in breast cancer treatment and prevention.

Abbreviations: CAT, chloramphenicol acetyltransferase; CI, combination index; CG, catechin gallate; EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; ER, estrogen receptor; ERE, estrogen response element; GTE, green tea extract; GC, gallocatechin; GCG, gallocatechin gallate.

Materials and methods

Reagents and cells

GTE was obtained from Pharmanex (batch no. 990222; Brisbane, CA) by proprietary methods (8). GTE concentration was expressed as μg/ml media

for *in vitro* experiments, and as g/l drinking water for animal experiments. EGCG, EC, ECG, β-D-glucuronidase type X-A from *Escherichia coli*, arylsulfatase type VIII from abalone entrails, estradiol-17β and tamoxifen were obtained from Sigma (St Louis, MO).

The human breast cancer cell lines MCF-7, ZR75, T47D and SKBR3 were obtained from American Type Tissue Culture Collection (ATCC, Rockville, MD). Cells were routinely maintained in RPMI medium 1640 (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). For estrogen-free experiments, medium was changed to phenol red-free RPMI 1640 with 0.5% dextran-coated charcoal (DCC) stripped FBS (Gemini Bio-Products, Woodland, CA). After 48 h, medium was changed to the same RPMI–DCC–FBS above, but containing estradiol-17β (1 nM) for 30 min before treatment with GTE and/or tamoxifen.

In vitro assay of proliferation

Cells were plated on to 48-well culture plates at 10 000 cells/well and incubated at 37°C in 5% CO_2 for 24 h in RPMI with 10% FBS media. The test sample was applied the following day. In the negative control wells, the growth media were changed to RPMI without FBS. On the third day, 1 μCi of [methyl-^{3}H]thymidine (Amersham, Piscataway, NJ) was added to each well. At 12–15 h later, plates were washed with phosphate-buffered saline (PBS). The cells were fixed with trichloroacetic acid, washed with ethyl alcohol and lysed with sodium hydroxide, using methods as described previously (8). After adding glacial acetic acid, the radioactivity of cell lysates was counted in scintillation solution (ScintiVerse, Fisher, Pittsburgh, PA). *In vitro* assays were performed in triplicates.

Mouse tissue catechin measurement

Nude mice were bred in a pathogen-free colony at our institution and 6-week-old female mice were used for this study. All mice were housed four per cage, and were fed *ad libitum* with sterilized food pellets and sterile water. Each group of four mice was assigned to (i) water, (ii) GTE 0.625 g/l water, (iii) GTE 1.25 g/l and (iv) GTE 2.5 g/l. After 4 days of treatment, the mice were killed by exposure to a nitrogen gas environment, and the mammary pads were removed.

Tissue polyphenol analysis was performed using a modification of the method of Lee *et al.* (12). About 100–200 mg of mammary tissue was homogenized in 0.8 ml of buffer A (40 mM NaH_2PO_4/0.1% EDTA, pH 5.8), mixed with 30 μl of buffer B (10% ascorbic acid–40 mM NaH_2PO_4/0.1% EDTA), 1500 U β-D-glucuronidase, 24 U sulfatase and 20 μl of 2.3 μM CG internal standard. The mixture was incubated at 37°C for 45 min. Mammary homogenates were extracted three times with 2 ml ethylacetate each. Aliquot of 10 μl of 0.2% ascorbic acid/0.005% EDTA was added to the pooled supernatant followed by vacuum concentration for 2 h at low heat using a Savant SC-100 Speed-Vac system (Savant Instruments, Farmingdale, NY). Samples were reconstituted in 200 μl of mobile phase A (75 mM citric acid and 25 mM ammonium acetate), and 20 μl aliquots were injected into the HPLC column. The column was eluted at room temperature with a linear gradient from 100% mobile phase A to 90% mobile phase A and 10% mobile phase B [75 mM citric acid–25 mM ammonium acetate/acetonitrile (50:50)] in 4 min at a flow rate of 1 ml/min. The gradient was linearly changed further to 70% A/30% B (4–12 min), 66%A/34%B (12–17 min), 63% A/37% B (17–20 min), 57% A/43% B (20–29 min), 100% B (29–33 min), 100% A (33–35 min) and maintained there until analysis of the next sample.

Mouse breast cancer xenografts

Six-week-old ovariectomized nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed four per cage, and were fed *ad libitum* with sterilized food pellets and sterile water. Mice were inoculated with estradiol-17β (1.7 mg/pellet; Innovative Research of America, Sarasota, FL) before the injection of 5×10^7 MCF-7 cells subcutaneously into the mid-back region. The tumor size was measured in three dimensions with calipers twice weekly starting at Day 7. Mice were observed for any change in behavior, appearance or weight. When tumors reached 30–50 mm^3, six animals were randomly assigned to each treatment group. Control mice drank water, and experimental mice drank GTE at the concentrations of 2.5 g/l and/or were inoculated subcutaneously with tamoxifen (20 mg/pellet; Innovative Research of America). At the end of the experiment at Day 64, the mice were killed and xenograft specimens were harvested for further analyses.

Immunohistochemistry

Paraffin-embedded tumor specimens were prepared as described previously (8). We used antigen retrieval with 0.01 M sodium citrate (pH 6.0) in a 95°C water bath for 20 min, and 1 mg/ml trypsin (Sigma) at room temperature for 3 min. Immunostaining was performed with the DAKO Envision peroxidase rabbit ready-to-use system. The slides were sequentially incubated at room temperature as follows: (i) in DAKO antigen block reagent to block non-specific antibody binding; (ii) with the rabbit anti-von Willebrand factor (vWf; Dako, Carpinteria, CA) primary antibody at 1:250 dilution for 1 h; (iii) with the DAKO secondary antibody to rabbit for 30 min; and (iv) developed with DAKO DAB (diaminobenzidine) solution. Positively stained vessels were counted in five separate fields for each specimen using an Olympus model BH2 microscope. Following the method established by Weidner (11), fields containing the highest density of vWf-positive vessels, i.e. 'bursts', were identified at scanning power and then counted at ×400 magnification. The numbers for the five fields were averaged.

ER immunohistochemistry was performed as above, without trypsin digestion. The primary antibody was used at 1:50 dilution (Biocare Medical, Walnut Creek, CA). The secondary anti-mouse antibody was from DAKO. The histological slides were reviewed by a Board-certified pathologist (J.Y.R.) and graded from 0 to 2+, using standard clinical criteria.

Apoptosis assay

Apoptosis was measured by the TUNEL (TdT-dUTP terminal nick-end labelling) assay. Cells were plated in the 2-well glass slides (Nalgen Nunc, Naperville, IL). After 48 h of incubation with GTE (5 or 10 μg/ml) and/or tamoxifen (1 or 10 nM), the ApopTag peroxidase kit (Intergen, Purchase, NY) was used to detect *in situ* apoptosis according to the manufacturer's protocol. Briefly, cells were fixed in paraformaldehyde, and post-fixed in ice-cold ethanol/acetic acid (2:1 v/v). After applying the equilibration buffer, the TdT (terminal deoxy-nucleotidyl transferase) was added, followed by anti-dioxigenin conjugate. Slides were stained with peroxidase substrate, counterstained by methyl green, dehydrated by xylene, and mounted under a glass coverslip to be viewed under the microscope at (400 magnification.

Paraffin-embedded specimens were cut into 5 μm sections, deparaffinized and then pretreated with proteinase K using the above kit.

Western blot analysis

After being maintained in estrogen-free conditions for 48 h, and with 1 nM estradiol-17β for 30 min, cells were treated with GTE and/or tamoxifen for 48 h. Cell lysates were centrifuged at 12 000 × *g* for 10 min, and the supernatant was retained. Protein concentration was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Approximately 50 μg of protein was separated by a 7.5% SDS–PAGE, and transferred on to a nitrocellulose membrane by electrophoretic blotting. The membrane was blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), and then incubated for 1 h with a 1:500 dilution of anti human ER-α affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were then washed three times over 1 h in TBST (TBS with 0.1% Tween-20), and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody goat anti-rabbit IgG (1:2000), and then washed in TBST as before. The membranes were developed using the Supersignal West Pico Chemiluminescent Western blotting detection system according to the manufacturer's instructions (Pierce, Arlington Heights, IL).

Analysis of estradiol binding

ER-α binding was determined by ligand binding with [^{3}H]estradiol-17β. Previous studies have shown that MCF-7 cells do not contain any significant levels of ER-β (13). Effects of GTE on specific estradiol binding in MCF-7 cells were determined as before (14). Briefly, MCF-7 breast cancer cells (1×10^6 cells/condition) were incubated with 2 nM [^{3}H]estradiol-17β (NEN, Boston, MA) and increasing concentrations of GTE (0.2–80 μg/ml). A 100-fold molar excess of unlabeled estradiol-17β was present with [^{3}H]estradiol-17β in paired samples for the determination of displaceable binding. Samples were incubated for 1 h at room temperature with vortex every 15 min. To stop the reaction, cells were placed on ice. Unbound steroids were removed with DCC, and counts per minute (c.p.m.) were determined by liquid scintillation counting.

ER transcriptional activation

ER transcriptional activity was assessed using an ERE–CAT (estrogen response element–chloramphenicol acetyltransferase) reporter gene. Transient transfections were performed in MCF-7 breast cancer cells with methods as before using the pERE-BLCAT reporter vector (14,15). In brief, cells in 60 mm Petri dishes were transfected using 2.5 μg of pERE-BLCAT vector and 0.5 μg of pCMV-β-galactosidase for transfection efficiency. Then, 12 μl of Fugene 6 (ROCHE Applied Science, Indianapolis, IN) was added per plate. Transfection was performed for 24 h in the presence of 1% DCC–FBS in phenol red-free RPMI. At 24 h after transfection, cells were treated

with vehicle alone, 1 nM estradiol-17β, 5 or 10 μg/ml of GTE, or combinations of these agents with tamoxifen. CAT reporter gene assay was performed after 18 h of treatment using the CAT-ELISA kit from Roche Applied Science. β-Galactosidase activity was assessed by using the β-Gal ELISA kit from Roche Applied Science for transfection efficiency.

p44/42 MAPK activity

Cells were maintained in estrogen-free conditions for 48–72 h before experiments, then treated with 2 nM estradiol in combination with selected inhibitors, such as 20 nM ICI 182,780 (an anti-estrogen agent; Astra Zeneca, Neward, DL), tamoxifen (10 nM) or GTE (40 μg/ml). Rapid effects of estradiol (and selected inhibitors) on phosphorylation and nuclear localization of MAPK (mitogen-activated protein kinase) were tested using confocal microscopy with breast tumor cells (16). For immunofluorescence studies with confocal microscopy, breast cells were grown on coverslips and then fixed with methanol as described previously (17). Cells were then immersed in primary phosphor-MAPK polyclonal antibody (anti-phospho-p44/p42 MAPK, Thr202/Tyr204, polyclonal antibody; New England Biolabs, Beverly, MA), and a fluorescent-labeled secondary anti-rabbit antibody.

Cell hybrid DNA was stained using Hochest 33258 dye at 0.5 μg/ml. After repeated washes with PBS, coverslips were mounted on glass microscope slides using mounting medium Fluoroguard Antifade Reagent (Bio-Rad) and viewed using a Leica TCS SP MP Inverted Confocal Microscope (Leica, Bannockburn, IL).

Aromatase activity

Aromatase activity in lung cancer cell lines was determined using the $[^3H]H_2O$ release method reported by Kinoshita and Chen (18). Breast cancer cell line SKBR3 was plated and grown to confluency in 6-well cell culture plates. Once confluent, cells were washed twice with PBS. To each well, 1 ml of serum-free medium containing substrate, 100 nM [1b-^{3}H]Androst-4-ene-3,17-dione (PerkinElmer, Boston, MA) and 500 nM progesterone (to inhibit 5α-reductase in cells) was added. The reaction was incubated for 3 h at 37°C. The reaction mixture was then removed and extracted with 1 ml of chloroform, vortexed and centrifuged for 10 min at a maximum speed of 10 000 × *g*. The upper aqueous layer was carefully removed and treated with 1 ml of dextran-treated charcoal. After centrifugation at maximum speed, supernatant containing the product, $[^3H]H_2O$, was counted in a liquid scintillation counter. Protein concentration was determined using the Bradford method, after dissolving cells in 0.5 M NaOH.

Statistical analysis

Descriptive statistics, such as mean and standard error, were used to summarize the results. The Student's *t*-test was used for univariate analysis, and the ANOVA test was performed for comparison among the various groups. Statistical significance was defined by $P < 0.05$.

For analysis to detect additive, synergistic or antagonistic effects, the multiple drug effect analysis method was used (19). Briefly, a combination index (CI) value was calculated to assess synergism or antagonism according to the following equation which assumes an independent mechanism of drug action (mutual exclusivity): $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D)_1$ and $(D)_2$ are the concentrations of Drug 1 and Drug 2 which combined produce *x*% inhibition, and $(D_x)_1$ and $(D_x)_2$ are the concentrations of each drug which alone produce *x*% inhibition. CI = 1 indicates an additive interaction, CI < 1 indicates synergy and CI > 1 antagonism.

Results

In vitro proliferation studies

The *in vitro* assays showed that individually, green tea or tamoxifen inhibited proliferation of human ER-positive MCF-7 breast cancer cells (Figure 1A and B). The inhibitory effects of these compounds were dose-dependent. The ID_{50} were as follows: 16.6 μg/ml of green tea and 1.2 μm of tamoxifen. We then tested the combination of green tea and tamoxifen. We used tamoxifen at a lower dose (nm) than the standard *in vitro* dose (μm), in order to better detect potential synergy. The combination of green tea and tamoxifen was more effective than either agent given alone (Figure 1C, $P < 0.05$). The CI calculations for 0.5–10 μg/ml green tea and 0.5–10 nm tamoxifen combinations consistently showed CI values <1, indicating synergism. We saw similar effects in two other ER-positive human breast cancer cells, ZR75 and T47D (Figure 1D–I).

Determination of catechin levels in mouse mammary fat pads

To determine whether green tea catechins attain measurable levels in breast tissues, we administered GTE in drinking water in a murine model. Each group of four mice was assigned to (i) water, (ii) GTE 0.625 g/l, (iii) GTE 1.25 g/l and (iv) GTE 2.5 g/l. After 4 days of treatment, the mice were killed, and mammary pads were then harvested for catechin measurement by HPLC methods. We chose Day 4, because previous investigators reported that murine plasma catechin levels are at maximal values at this time interval (20). We observed that levels of catechins in murine mammary fat pads increased with higher amounts of tea administered (Figure 2A). EGCG was the catechin type with the highest concentration in breast tissues, followed by EC, then ECG.

Mouse xenograft studies

We further tested the combination of green tea and tamoxifen, using MCF-7 breast cancer cells *in vivo*. In the mouse xenograft model, either green tea or tamoxifen given alone inhibited the growth of MCF-7 cells in nude mice: 341.1 ± 48.8 mm^3 in green tea-treated and 177.8 ± 37.6 mm^3 in tamoxifen-treated, versus 622.2 ± 163.3 mm^3 in control animals at Day 64 (Figure 2B). We also observed that dual therapy with green tea and tamoxifen elicited a more profound anti-tumor effect than either agent given alone (116.5 ± 31.9 mm^3, $P = 0.0035$, ANOVA test). There was no weight difference between the animal groups, and we observed no toxicity.

The xenograft tumors were further analyzed for the extent of angiogenesis, necrosis and apoptosis. The histological slides were reviewed by a Board-certified pathologist (J.Y.R). The combination of green tea and tamoxifen significantly decreased the overall blood vessel density in mouse tumors [16.5 ± 1.5 vessels/high power field (HPF)], compared with the green tea-treated group (21.9 ± 1.3 vessels/HPF), the tamoxifen-treated group (21.3 ± 3.2 vessels/HPF) and the control group (30.7 ± 2.2 vessels/HPF, $P = 0.023$, ANOVA; Figure 3A). With H&E analysis, there were no significant differences in mitosis among the four mouse groups (Figure 3B). However, individual green tea and tamoxifen treatments resulted in larger areas of tumor necrosis, 16.7 ± 3.6 and 16 ± 4, respectively, versus 6.7 ± 1.1 in controls ($P < 0.0001$, ANOVA). Overall, mice that received both green tea and tamoxifen had the most necrotic tumors (23 ± 5.4, $P < 0.0001$, ANOVA). Apoptosis was measured both histologically with H&E staining and with the TUNEL method. We observed the greatest degree of tumor cell apoptosis, as measured by the TUNEL method, in the group that received green tea and tamoxifen in combination ($P = 0.0208$, ANOVA; Figure 3C).

We then carried out similar TUNEL analyses with MCF-7 cells in culture in order to confirm the apoptosis effect. Again, we observed the most significant level of cellular apoptosis with the combination of green tea and tamoxifen treatment ($P = 0.0271$, ANOVA; Figure 4A). Similar data were obtained with two other ER-positive human breast cancer cells, ZR75 and T47D (Figure 4B and C).

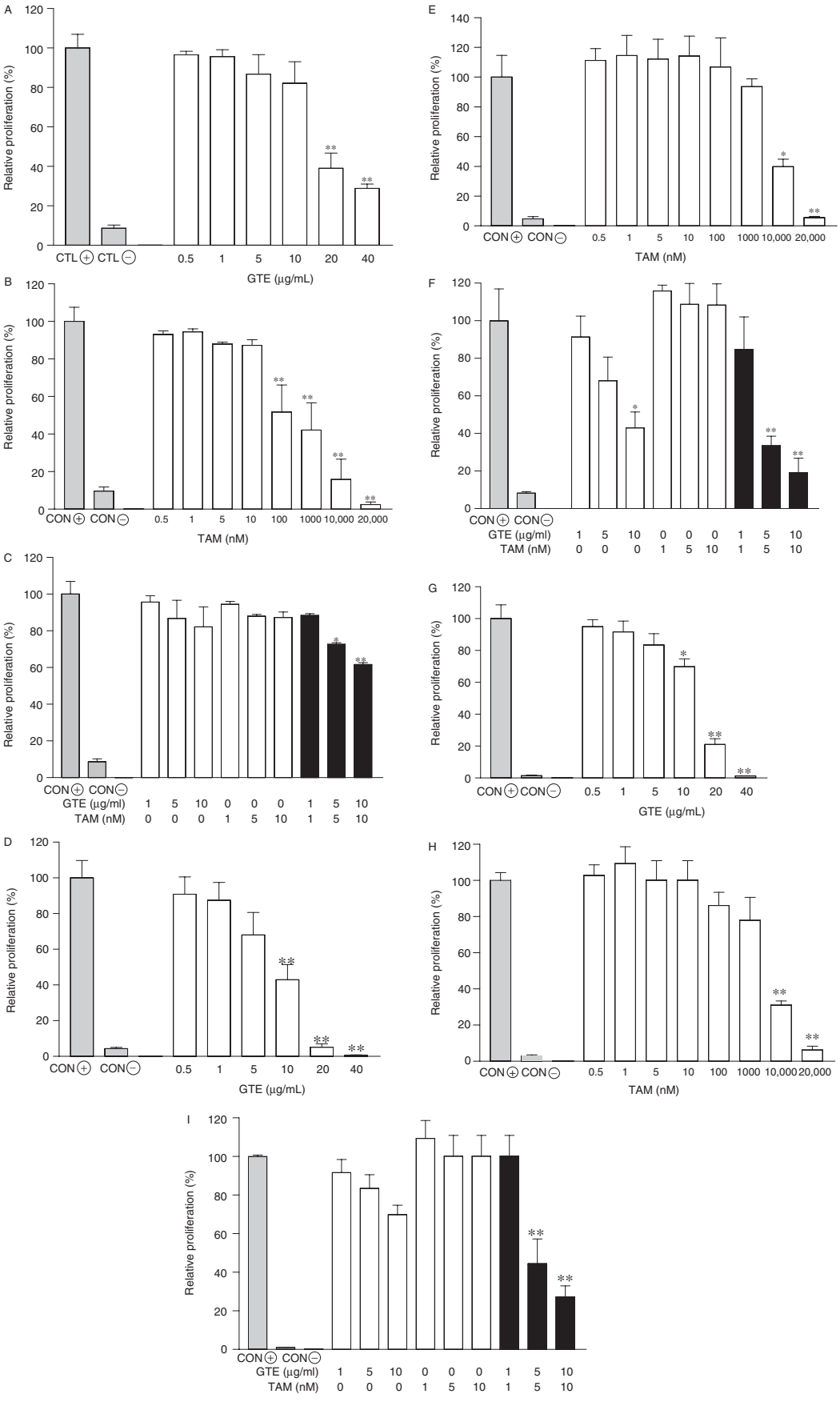

Fig. 1. Effect of GTE and tamoxifen (TAM) on human breast cancer cell proliferation. (A–C) MCF-7, (D–F) ZR75 and (G–I) T47D cells. Exposure for 2 days to (**A, D** and **G**) GTE alone, (**B, E** and **H**) TAM alone, and (**C, F** and **I**) GTE and TAM combination. The combination of GTE and TAM is more effective than either agent alone. $*P < 0.05$, $**P < 0.01$, positive control (CON+), negative control (CON−).

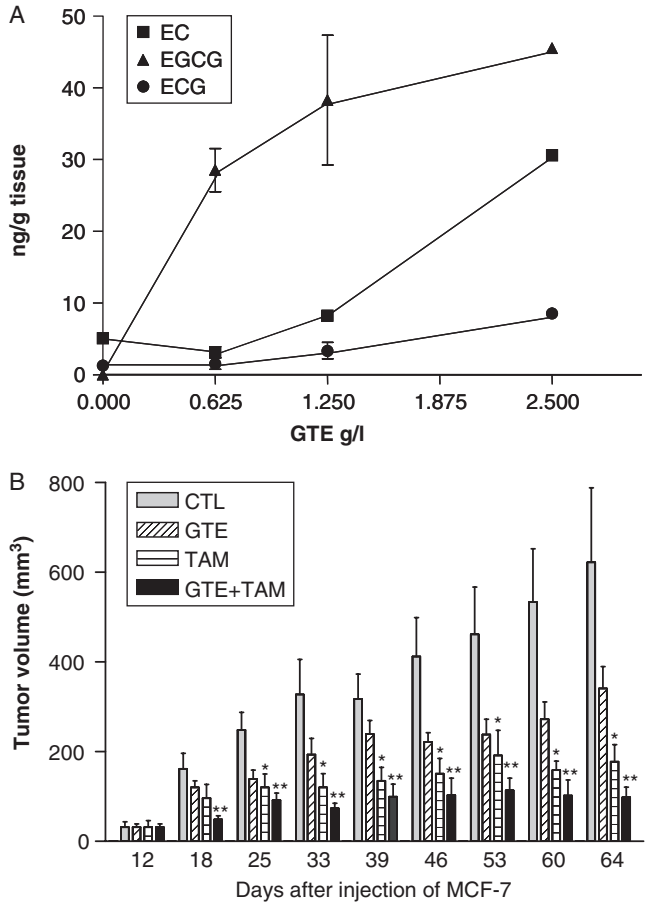

Fig. 2. (**A**) Analysis of catechin levels in the mammary fat pads of mice given GTE. Mice drank water with different doses of GTE for 4 days, then killed and their mammary fat pads were harvested. Their contents of EGCG, EC and ECG were determined by HPLC. (**B**) Effect of GTE and tamoxifen (TAM) on MCF-7 breast cancer xenografts. Mice were injected subcutaneously with 5×10^7 MCF-7 cells. The tumor size was measured in three dimensions with calipers and expressed as volume. The combination of GTE and TAM is more effective than either agent alone. $^*P < 0.05$, $^{**}P < 0.01$, untreated control (CTL).

Green tea effects on ER-α levels and specific binding of estradiol

To explore the effect of the treatment regimens on the expression of human ER-α, we performed immunohistochemical studies on the mouse xenografts above. The combination of green tea and tamoxifen significantly reduced the levels of ER-α (0.2+ ± 0.2), in comparison to either agent alone (1.25+ ± 0.25 for green tea and 0.5+ ± 0.29 for tamoxifen), and in comparison to controls (1.33+ ± 0.33, $P = 0.0081$, ANOVA; Figure 3D). This phenomenon was reproduced *in vitro* by use of western blot analyses of MCF-7 cells (Figure 4D). Cells that received 10 μg/ml GTE and 1 nM tamoxifen had only 42.3 ± 10.2% of control ($P < 0.0001$, ANOVA), and those that received 10 μg/ml GTE and 10 nM tamoxifen 24.9 ± 5.6% of control ($P < 0.0001$, ANOVA). Interestingly, we observed that green tea alone can exert a suppressive influence on the levels of ER-α proteins, in a dose-dependent manner (54.1 ± 4.2% of control with 10 μg/ml GTE, $P < 0.0001$, ANOVA). We saw similar effects in two other ER-positive human breast cancer cells, ZR75 and T47D (Figure 4E and F).

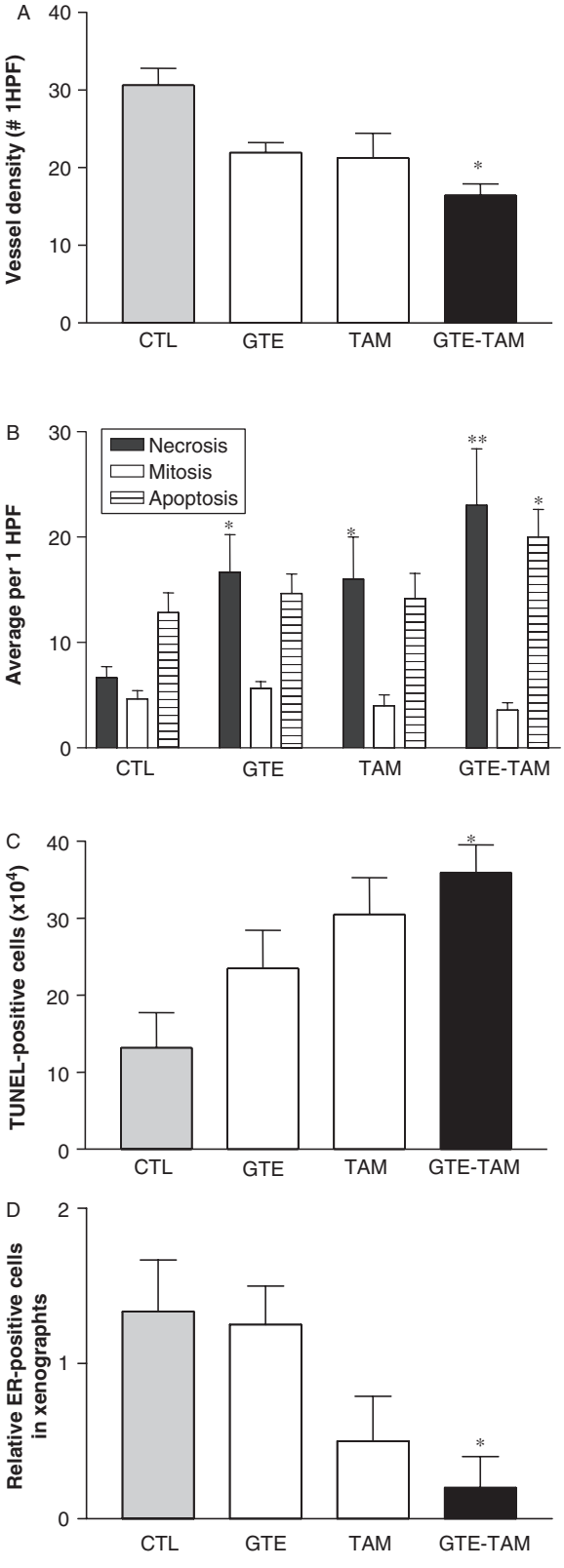

Fig. 3. Analysis of MCF-7 breast cancer xenografts. Tumor specimens harvested after 52 days of treatment were (**A**) stained with vWf (von Willebrand factor) antibody, and vessel density per HPF (×400) presented. (**B**) Specimens were stained with H&E; and necrosis, mitosis and apoptosis were quantified histologically. (**C**) Apoptosis was determined with the TUNEL assay. (D) Specimens were stained with ER-α (estrogen receptor) antibody. The combination of GTE and TAM is more effective than either agent alone. $^*P < 0.05$, $^{**}P < 0.01$, GTE, tamoxifen (TAM), untreated control (CTL).

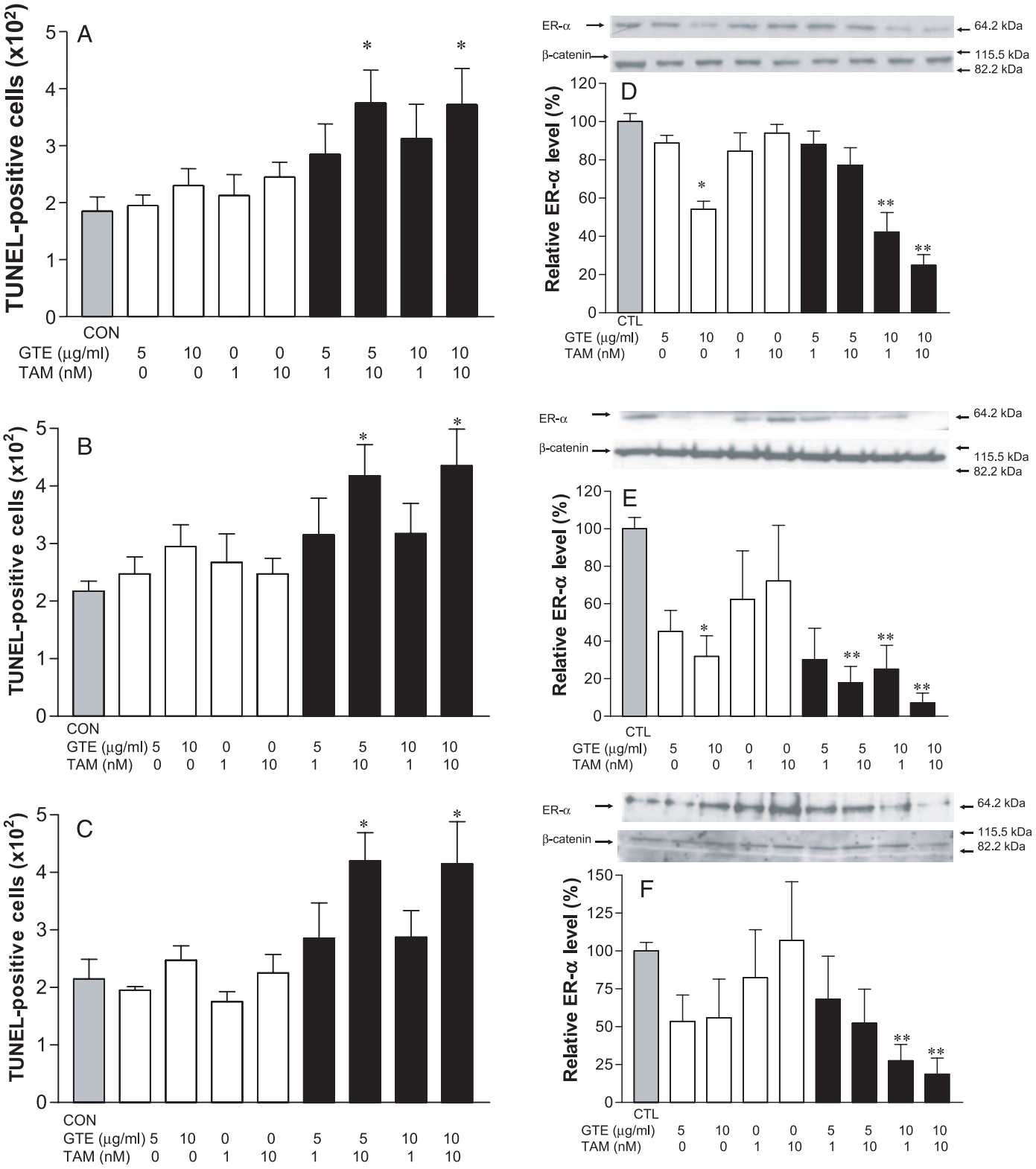

Fig. 4. Effects of GTE and tamoxifen (TAM) on human breast cancer cells. Apoptosis was determined with the TUNEL assay: (**A**) MCF-7, (**B**) ZR75 and (**C**) T47D cells. Western blot analysis of cell lysates with anti-ER-α antibody; ER-α levels were normalized by expression of β-catenin: (**D**) MCF-7, (**E**) ZR75 and (**F**) T47D cells. The combination of GTE and TAM is more effective than either agent alone. $^{*}P < 0.05$, $^{**}P < 0.01$, untreated control (CON).

As reported previously, we found that MCF-7 human breast cancer cells show specific and saturable binding of [^{3}H]estradiol-17β (14). Using this specific estrogen-binding assay, incubation of cells with GTE at concentrations ranging from 0.2 to 80 μg/ml was performed in order to estimate competition for specific estrogen-binding sites. No significant competition for specific estrogen binding was detected at concentrations ranging from 0.2 to 40 μg/ml. However, at the

dose of 80 μg/ml, green tea did elicit a small reduction in estrogen binding (Figure 5A).

Effects of green tea on ER-dependent transcription

We examined ER transcriptional activity on a consensus ERE that includes the commonly used *Xenopus* vit A2 ERE palindrome. To determine the effects of GTE on transactivation of ER, we used a reporter plasmid, pERE-BLCAT, containing a palindromic ERE and the CAT reporter gene. Treatment with estradiol-17β-induced transactivation of the ERE-CAT reporter in MCF-7 breast cancer cells transfected with pEREBLCAT by ∼1.2-fold (Figure 5B). GTE (10 μg/ml, $P < 0.05$) inhibited estrogen-induced ERE transactivation. Moreover, co-administration of tamoxifen in combination with GTE resulted in ∼50% inhibition of transcription of the ERE-CAT reporter gene ($P < 0.001$). These results demonstrate that combinations of tamoxifen and GTE are more effective at suppressing transcription mediated by an ERE than either compound alone. We observed a similar phenomenon with ZR75 cells (Figure 5C).

Effects of green tea on estrogen-induced stimulation of p44/42 MAPK activity

Post-receptor signal transduction events, such as activation of MAPK, may contribute to the proliferative effects of estrogen in breast cells. Thus, we assessed acute effects of GTE and estrogen on MAPK phosphorylation. The effect of estradiol on activation of p42/p44 MAPK was assessed at 30 min in MCF-7 cells treated with control, estrogen (2 nM) alone or one of several different anti-estrogens or GTE (40 μg/ml). Treatment with estradiol alone elicited an increased phosphorylation of MAPK as evidenced by prominent staining with anti-phospho-MAPK antibody as compared with control preparations. In addition, a marked translocation of MAPK to the nucleus was induced by estrogen (Figure 6A). Both ICI 182,780, a pure anti-estrogen, and tamoxifen effectively blocked the estrogen-induced activation of MAPK. This was evident by reduced phosphorylation of MAPK and by reduced nuclear translocation of MAPK. Furthermore, GTE was also very effective in disrupting these estrogenic actions, and combination of GTE with tamoxifen appeared to elicit a greater inhibitory effect than that found with tamoxifen alone. Since MAPK plays a critical role in regulating the proliferation of breast cells, this activity of GTE could be important in modulating breast tissue growth. Moreover, the results suggest that GTE may alter downstream MAPK signaling, an effect that can impact both ER-positive and ER-negative cell types. We saw similar effects in ZR75 and T47D cells (Figure 6B and C).

Green tea has no effect on aromatase activity

The treated SKBR3 cells were incubated with GTE (40 μg/ml) for 48 h. Untreated SKBR3 cells were used as control. The reaction was repeated in triplicates for both conditions. Aromatase activity was determined in both treated and control cells: 1.12 ± 0.11 versus 1.34 ± 0.13 pmol/mg/h, respectively. There was no significant difference between treated and control SKBR3 breast cancer cells.

Discussion

To our knowledge, this study is the first to provide mechanistic evidence that the combination of green tea and

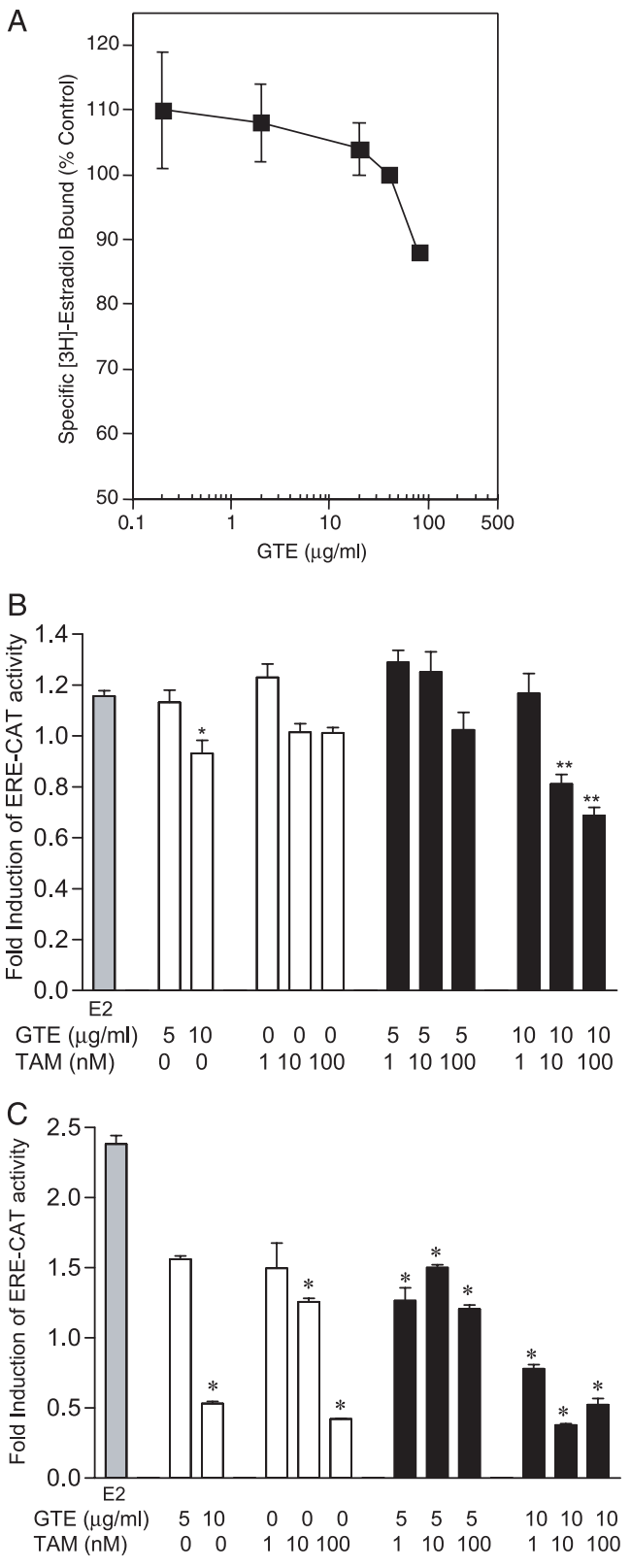

Fig. 5. (**A**) Effect of GTE on competitive displacement of [^{3}H]estradiol-17β binding in MCF-7 breast cancer cells. Data are shown as percentage of appropriate control levels. No significant competition for specific estrogen binding is detected at concentrations ranging from 0.2 to 40 μg/ml. Effect of GTE on ERE-CAT activity. Treatment with estradiol-17β (E_2)-induced transactivation of the ERE-CAT reporter in MCF-7 (**B**) and ZR75 (**C**) breast cancer cells transfected with pEREBLCAT by ∼1.2-fold. Co-administration of tamoxifen in combination with GTE resulted in ∼50% inhibition of transcription of the ERE-CAT reporter gene ($P < 0.001$).

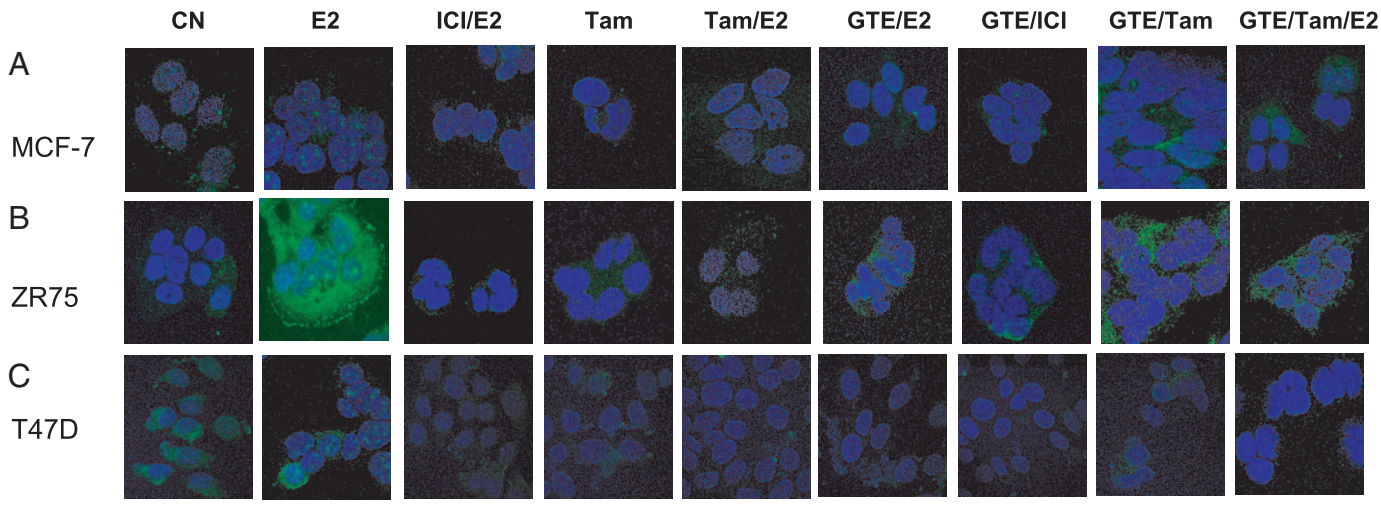

Fig. 6. Effects of GTE and tamoxifen (TAM) on estrogen (E_2)-induced phosphorylation and nuclear localization of p42/p44 MAPK in MCF-7 (**A**), ZR75 (**B**) and T47D (**C**) breast cancer cells. Control (CN) cells show minimal activation (phosphorylation; green or bright fluorescence due to stain with anti p-MAPK antibody). E_2 treatment shows marked MAPK activation and nuclear localization. This E_2-induced MAPK activation is blocked by an anti-estrogen agent ICI 182,780, tamoxifen and GTE.

tamoxifen is more potent than either agent alone in suppressing ER-positive breast cancer growth. This improved inhibition occurs via direct anti-proliferative and pro-apoptotic effects on tumor cells *in vitro* and *in vivo*. In mouse tumor models, suppression of angiogenesis is translated to larger areas of necrosis and lower blood vessel density in the treated xenografts. Furthermore, green tea appears to add to the effectiveness of tamoxifen, both at the level of the estrogen receptor in the tumor cells as well as in direct effects on the estrogen-induced signaling.

There is good precedent that multiple drugs in breast cancer may be more effective than single agents. For example, current regimens routinely include multiple drugs (1). There is also preclinical evidence that the combination of an anti-angiogenic agent with a chemotherapeutic drug can result in additive or synergistic effects (21). Furthermore, a recent report showed that green tea polyphenols may inhibit the multidrug resistance P-glycoprotein activity, which is responsible for much of the resistance to chemotherapeutic drugs (22). EGCG has also been observed to synergize with sulindac to prevent colon carcinogenesis in rats (23), and with tamoxifen on the lung cancer cell line PC-9 (24). A recent publication reported synergistic *in vitro* cytotoxicity of 4-hydroxytamoxifen and EGCG in ER-negative breast cancer MDA-MB231 cells, but not in ER-positive MCF-7 cells (25). Our study used total GTE and tamoxifen in a different assay system, which may account for different experimental results.

Flavonoids in green tea are among the dietary factors that may play a role in cancer protection and have been shown to have potent antioxidant effects (26). Green tea has also been found to block certain steps in carcinogenesis. Green tea inhibits the formation of PhIP (2-amino-1-methyl-6-phenylimidazo pyridine)-induced DNA adduct formation, which leads to reduced tumorigenicity in animal chemoprevention studies (27,28). It also inhibits the activity of transcription factors, AP-1 and NF-κB, and the synthesis of nitric oxide (29). EGCG reduces the proliferation and induces apoptosis of cancer cells (30). Binding of EGF (epidermal growth factor) to its receptor is suppressed by EGCG (31), and IGF-1 (insulin-like growth factor-1) signaling is similarly altered (32,33). Researchers have shown that green tea can upregulate tumor suppressor genes (34). Green tea may also inhibit HER-2/neu signaling in breast cancer cells (35). Other effects are reported on cell cycle progression (36) and MAPK activity (37).

Recently, green tea was reported to inhibit angiogenesis using both *in vitro* and *in vivo* assays (38,39). Studies in our laboratory have suggested that GTE inhibits breast cancer growth via a direct anti-proliferative effect on tumor cells, as well as by indirect effects on endothelial cells (8). We found that green tea reduces levels of two important angiogenic factors, VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor), as well as aFGF (acidic fibroblast growth factor) in breast cancer (9,10). Others have noted effects of green tea on additional angiogenesis-related molecules, including IL-8 (interleukin-8), urokinase, matrix metalloproteinases (MMP-2 and MMP-9) (40), PDGF (platelet derived growth factor) (41) and TNF-α (tumor necrosis factor) (42). Angiogenesis is a crucial process in malignancy and may account for, in part, the cancer-preventive effect of green tea *in vivo*.

In the clinic, endocrine therapy is an important intervention for women with breast cancers that express ER, and treatment with tamoxifen has enhanced patient survival (2). The success of endocrine therapy is dependent on tight regulation of breast cell growth by steroid receptors (43). However, as breast cancer progresses, it usually becomes resistant to anti-estrogens, and most patients eventually stop responding to tamoxifen therapy. New options for endocrine therapy are urgently needed to reverse this outcome. Targeting this signaling axis may promote introduction of more effective and less toxic anti-hormone treatments for human breast cancers (14,44). Recently, aromatase inhibitors have become a possible alternative to tamoxifen (45). Our preliminary results with green tea showed no significant aromatase activity inhibition in an *in vitro* assay. Other investigators have reported modest aromatase suppression by green tea (46) and black tea (47). The issue whether or not green tea has a role in supplementing aromatase inhibitor therapy deserves further studies.

Little is known about the effect of green tea on the estrogen signaling pathway in breast cancers (48–51).

However, the present findings suggest that GTE may elicit downregulation of ER and disruption or ER-dependent transcription. Further, the herbal extract shows inhibitory effects on early estrogen-induced changes in the activity and subcellular localization of MAPK, an effect that may be important in cell growth regulation. Importantly, these effects of GTE are enhanced by combined treatment with tamoxifen. Further understanding of the interactions between green tea and ER signaling is crucial for the use of this natural product to treat or prevent ER-positive breast cancers, which represent the majority of primary breast tumors. The results of these experiments offer some new insight, and will help in the design of future preclinical and human trials. If green tea is confirmed to have anti-tumor activity alone or in combination with tamoxifen, targeting of this herbal product may help to improve the outcome of this common and deadly disease.

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