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Breast Cancer Prevention by Green Tea Catechins and Black Tea Theaflavins in the C3(1) SV40 T,t Antigen Transgenic Mouse Model Is Accompanied by Increased Apoptosis and a Decrease in Oxidative DNA Adducts

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Tea consumption is associated with a reduced risk of mammary cancer as reflected by epidemiological studies and experiments in carcinogen-induced rodent models of mammary carcinogenesis. We tested the hypothesis that green tea catechins (GTC) or theaflavins from black tea (BTT) interfere with mammary carcinogenesis in C3(1) SV40 *T,t* antigen transgenic multiple mammary adenocarcinoma (TAg) mice and that GTC/BTT affect tumor survival or oxidation status. TAg mice received GTC/BTT (0.05%) in drinking water for their lifetime. As compared to control mice, they survived longer and had smaller tumors. On microscopic inspection, the size of the largest tumor per mouse was decreased by 40−42% ($p < 0.01$). GTC (0.01%) and BTT (0.05%) increased levels of cleaved caspase 3 in tumor tissue by 67 and 38%, respectively ($p < 0.05$), intimating increased apoptosis. Tumor levels of the malondialdehyde−DNA adduct M_1dG in mice receiving GTC or BTT (0.05%) were reduced by 78 ($p < 0.001$) or 63% ($p < 0.05$), respectively, as compared to controls. The results render the exploration of the breast cancer chemopreventive properties of tea preparations in humans worthwhile.

KEYWORDS: Chemoprevention; catechins; theaflavins; breast cancer; malondialdehyde

INTRODUCTION

Breast cancer is the third most common cause of cancer-related deaths worldwide, and incidences continue to increase despite the introduction of national screening programs and awareness campaigns (*1*). This is particularly true for ductal carcinoma in situ (DCIS), which accounts for approximately 14% of breast cancer incidence in the United States (*2*, *3*). Currently, no strategy exists for the prevention of recurrence or disease progression of DCIS. Evidence for the notion that breast cancer is preventable comes from epidemiological data (*4*), especially that of migrants (*5*). Breast cancer incidence rates in Japan and China are significantly lower than in the United States (*4*), and Western countries have a four-fold increased incidence as compared to less industrialized nations (*4*, *6*). Migrants, who go from low risk areas to regions with a high risk, adopt the incidence rates of the host nation within two generations (*7*). Diet has been associated with the causation and prevention of breast cancer (*8*), and there is epidemiological evidence to suggest that the lower incidences of breast cancer in Japan and China are related to the consumption of green tea (*9*). Furthermore, recurrence of breast cancer was significantly decreased in Japanese women consuming three or more cups of green tea per day (*10*). Polyphenolic catechins, in particular epigallocatechin-3′-gallate (EGCG), are thought to be responsible for the chemopreventive efficacy of green tea (for reviews, see refs *11* and *12*). The majority of tea intake across the world is in the form of black tea. Black tea is produced from green tea by fermentation, during which catechins undergo oxidation to theaflavins and thearubigins. While the epidemiological evidence for breast cancer prevention in populations consuming black tea is inconclusive (*13*), black tea extracts have demonstrated chemopreventive activity in rodent models of breast carcinogenesis (*14*−*16*). The catechin content of green and black tea varies, with concentrations in the range of 30−42 and 3−9% (of extracted tea solids), respectively. Black tea contains 2−6% theaflavins, i.e., theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3, 3′-digallate. The latter of these cogeners is thought to be pharmacologically the most potent (*17*−*19*). Theaflavins reduce the survival of human breast cancer-derived cells in vitro, with a potency comparable to that of EGCG (for a review, see ref *17*). Limited bioavailability in vivo (*20*) implies that theaflavins may play a lesser role in cancer chemoprevention

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by tea extracts than tea catechins, but no direct comparison has so far been undertaken.

We wished to compare the breast cancer chemopreventive efficacy in vivo of green and black tea extracts side-by-side by employing mixtures of catechins (GTC, green tea catechins) and theaflavins (BTT, black tea theaflavins). The rodent model chosen for comparison of these extracts is the C3(1)SV40 *T,t* antigen transgenic multiple mammary adenocarcinoma mouse (TAg). In TAg mice, expression of the SV40 transforming sequences (*T* and *t* antigen) is targeted to the mammary epithelium by a fragment of the rat prostatic steroid binding protein promoter C3(1) (*21*). The *T*-antigen binds and functionally inactivates *p53* and *Rb* tumor suppressor genes (*22*, *23*). The consequent perturbation of cell homeostasis is thought to be responsible for mammary carcinogenesis, and all female TAg mice develop palpable tumors (*21*). This rodent model is considered a suitable model of the human disease, because the murine mammary tumors progress from estrogen receptor positive to negative status (*24*), and in terms of histology, the intraduct carcinomas resemble human DCIS (*25*).

Tea catechins and theaflavins possess strong antioxidant properties (*19*, *26*) thought to contribute to their cancer chemopreventive efficacies. Oxidative stress plays a key role in carcinogenesis (*27*), and increased lipid peroxidation is associated with early stages of carcinogenesis (*28*). Malondialdehyde is an endogenous product of lipid peroxidation that can induce mutagenesis through the formation of a DNA adduct (*29*). Levels of the malondialdehyde−DNA adduct 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)one (M_1dG) were found to be significantly higher in breast tissue from cancer patients than in equivalent tissue from healthy women (*29*). The level of M_1dG has been employed as an indicator of oxidative damage and coincidentally as a measure of antioxidant activity (*29*). Here, we tested the hypothesis that M_1dG can serve as a marker of the breast cancer chemopreventive efficacy of the tea extracts.

There is a growing realization that induction of apoptosis in neoplastic or preneoplastic cells plays an important role in the chemopreventive efficacy of some diet-derived agents including catechins and theaflavins (*30*). Therefore, we explored whether tea preparations affect levels of cleaved caspase 3 in mammary tumor tissue. Cleavage of caspase 3 to its 19 and 17 kDa fragments is one of several characteristic hallmarks of apoptosis mediated via the mitochondrial pathway (*31*). Furthermore, the hypothesis was tested that tea polyphenols can be detected by high-performance liquid chromatography (HPLC)−mass spectrometric analysis in plasma and/or tumor tissue of TAg mice after exposure to GTC or BTT. Overall, the experiments were designed to directly compare the chemopreventive efficacies of GTC and BTT and to contribute to the body of evidence, which will determine whether it is appropriate to evaluate green and/or black tea extracts for the chemoprevention of mammary cancer in humans.

MATERIALS AND METHODS

Tea Preparations and Reagents. The green tea extract (GTC) used in this study was purchased from Hunan King Long Bio-Resource Co. Ltd. (Xingsha, Changsha, Hunan, China) and was comprised of 95% polyphenols including 3% epicatechin, 10% epigallocatechin, 5% epicatechin-3-gallate, and 60% epigallocatechin-3-gallate. Black tea extract (BTT) was provided by Unilever R&D (Colworth House, Sharnbrook, United Kingdom) and contained 11% theaflavin, 28% theaflavin-3-gallate, 16% theaflavin-3′-gallate, and 45% theaflavin-3,3′-digallate. The caffeine contents of GTC and BTT were <0.5 and 0%, respectively. The composition of GTC and BTT was checked by HPLC analysis.

Animals and Cells. Experiments were carried out under animal project license PPL 40/2496, granted to Leicester University by the UK Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCCR guidelines (*32*). A breeding colony was established with wild-type FVB mice and female and male C3(1) SV40 *T,t* antigen (TAg) mice on an FVB background; the latter was purchased from the Jackson Laboratory (Bar Harbor, ME). Ear tissue from newborn mice was genotyped for the presence of the transgene using polymerase chain reaction (PCR) as described previously (*21*).

Human-derived MDA-MB-468 breast carcinoma cells were used in orientation experiments to explore the effect of tea preparations on cell survival and M_1dG levels. GTC and BTT were dissolved in water and sterile-filtered, and solutions were diluted with medium. Cells (tested negative for mycoplasma infection) were routinely cultured in RPMI medium supplemented with 10% fetal calf serum and 2 mM glutamax and were used after 20−30 passages. For assessing M_1dG levels, cells (10^6 per well) were incubated for 24 h with or without GTC/BTT at concentrations of 3.1−25 μg/mL. For assessment of the effect of tea preparations on cell number, cells (1×10^4 per well) were counted after incubation with GTC/ BTT for 48 h using a ZM model Coulter Counter (Beckman Coulter, Luton, United Kingdom).

Intervention and Evaluation of Tumor Development. Heterozygous transgenic female mice aged 4 weeks were randomly assigned to control and treatment groups (group size, 10−16). Mice received either GTC or BTT with their drinking water either at 0.01 or 0.05% (w/v), and controls received drinking water alone. Solutions of GTC and BTT were freshly prepared three times a week. From 11 weeks of age, mice were examined once to twice weekly for the presence of tumors by palpation. The tumor size was measured using calipers. The tumor volume was calculated using the equation:

$$V = \frac{[D \times (d^2)]}{2}$$

where *D* represents the long and *d* the short diameter. Three study designs were used. First, animals were exposed to tea preparations from week 4 to the end of the experiment, and they were killed (by cervical dislocation) when the tumor size, as determined by the sum of the length of palpable tumors per mouse, exceeded 17 mm (study 1). Second, this experiment was repeated, and tumors were dissected and flash frozen in liquid nitrogen prior to storage at −80 °C (study 2). Third, animals received tea until they were 19 weeks of age, after which they were killed, so that carcinogenesis could be compared at a fixed age (study 3). Animals were shaved, and the pelt was removed, weighed, and fixed in buffered formalin. Blood was collected at the end of each experiment by cardiac puncture and centrifuged (7000*g* × 10 min). Plasma and leukocytes were stored at −80 °C until analysis.

Pathology. After 2 weeks of fixation in formalin, the mouse pelt was cut to yield five transverse blocks corresponding to the five pairs of mammary glands. These were embedded conventionally in paraffin wax. Sections (5 μm) were cut and stained with hematoxylin and eosin. Neoplasms were assessed on the basis of the largest lesion per block.

Measurement of M_1dG. M_1dG was measured in MDA-MB-468 cells, which had been incubated with tea preparations, and in white blood cells and tumor tissue from mice, which had received GTE or BTT. White blood cells were collected after centrifugation of pooled blood from two to three mice. Mammary tumors were individually dissected from the mammary pelts of mice. MDA-MB-468 cells or leukocytes were centrifuged (100*g*). Pelleted cells and tumor tissues were digested with Proteinase K (Qiagen, Crawley, United Kingdom) and RNAse A (100 μg/mL for cells and 100 mg/mL for tumor tissue; Sigma). DNA was extracted from cells or tissue as described by the manufacturer's instructions (Qiagen handbook for Genomic DNA Extraction Kit). The DNA content was determined by UV absorbance. M_1dG was quantified by immunoslot blot, using a primary anti-M_1dG antibody provided by Dr. L. Marnett (Vanderbilt University, TN) as reported by Leuratti et al. (*33*).

Immunohistochemical Staining for Proliferating Cell Nuclear Antigen (PCNA). Paraffin-embedded sections were obtained from the

largest lesion in each mouse (study 3). Sections were deparaffinized, immersed in citrate buffer, microwaved to retrieve antigen, and treated with hydrogen peroxide (3%) to inhibit endogenous peroxidase. Sections were then washed and stained with the primary PCNA antibody (one in 50 dilution, NCL-PCNA, Novocastra, United Kingdom) followed by exposure to a peroxidase-labeled goat anti-mouse IgG2a secondary antibody (Serotec, Oxford, United Kingdom). PCNA-stained nuclei were visualized using 3,3′-diaminobenzidine (1 mg/mL) and counterstained with hematoxylin. The numbers of stained nuclei in five fields per tumor section were counted (40× magnification). The PCNA labeling index (%) was calculated by dividing the number of stained nuclei by the total number of nuclei × 100.

Measurement of Annexin V FITC in Cells and Cleaved Caspase 3 Levels in Tumors. In an orientation experiment, induction of apoptosis by tea preparations was explored in MDA-MB-468 cells. Cells (1×10^5) were incubated with BTT/GTE for 24 and 48 h, harvested, washed with phosphate-buffered saline, and incubated with Annexin V FITC conjugate and propidium iodide (both from Caltag Medsystems, Buckingham, United Kingdom) following the manufacturer's protocol. Stained cells were determined by fluorescence-activated cell sorting (Becton Dickinson FACscan, BD Biosciences, Oxford, United Kingdom). Apoptotic cells (stained with Annexin V FITC conjugate) were those in the upper left quadrant of the fluorescence-activated cell sorting histogram; viable "normal" cells were those in the lower left quadrant. Cells in the upper right quadrant represented late apoptotic or necrotic cells. For Western blot analysis of cleaved caspase 3, tumor tissue (~50 mg) was homogenized in ice-cold lysis buffer (120 mM NaCl, 50 mM tris base, 0.5% Triton X-100, 1 mM EDTA and EGTA, and 1 mM protease inhibitor Na_3VO_4). Protein lysates were stored (−20 °C), and the protein concentration was determined using the Bradford assay. Aliquots (100 μg) of protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated with milk powder (5 or 10%, w:v, in 1 M tris-buffered saline:tris base, pH 7.5, 0.9% NaCl, and Tween-20). Membranes were incubated with a cleaved caspase 3 primary antibody (Cell Signaling Technology, Danvers, United States) for 12 h at 4 °C. After they were washed, the membranes were incubated with anti-rabbit IgG horse radish peroxidase (HRP) secondary antibody (Cell Signaling Technology) for 1 h at room temperature. After further washes, protein expression was detected by chemiluminescene (ECL Western blot detection reagent; Amersham Biosciences, Chalfont, United Kingdom) and exposed to sensitive film. To confirm equal protein loading and transfer, blots were stripped, reprobed for β-actin using an anti-actin rabbit polyclonal antibody (Cell Signaling Technology), and incubated overnight at 4 °C, after which they were exposed to anti-rabbit IgG HRP secondary antibody. Detection was by chemiluminescence as described above. The band intensity was quantified by densitometry using a transilluminator (Gene Genome Bioimaging System, Syngene, Cambridge, United Kingdom), adjusting for differences in protein loading.

HPLC-Mass Spectrometric Analysis of Catechins and Theaflavins. Mammary tumor tissue was homogenized (1:2.5) in sodium phosphate buffer (0.4 M) containing ascorbic acid (20%) and EDTA (0.1%) using a blade homogenizer. Aliquots (200 μL) of plasma or tumor homogenate were extracted with ice-cold aqueous acetone:acetic acid (9:1, 1 mL). Prior to extraction, some biomatrix samples (200 μL) were incubated with β-glucuronidase (100 μL) from *Helix pomatia* (Sigma-Aldrich, 45 min, 37 °C) to hydrolyze glucuronide and sulfate conjugates. Samples were kept at −20 °C for 10 min, and precipitated protein was removed by centrifugation (7000*g*, 10 min, 4 °C). The supernatant was dried under nitrogen and resuspended in mobile phase A (25 μL, see below) containing 5% ascorbic acid and 0.1% EDTA.

Separation and identification of catechins and theaflavins were achieved using an Applied Biosystems MDS Sciex API2000 LC/MS/MS system (Applied Biosystems, Warrington, United Kingdom) with sample delivery through an Agilent 1100 series HPLC (Agilent Technologies UK Ltd., South Queensferry, United Kingdom). HPLC separation, which constitutes a modification of a previously reported method (*34*), was achieved on a Xterra phenyl column (2.1 mm × 150 mm, 3.5 μm) in line with a guard column (2.1 mm × 20 mm, 3.5 μm, both from Waters Co., Elstree, United Kingdom) and a two-component mobile phase (A, 5 mM ammonium acetate, pH 3.0, 1.75% acetonitrile, and 0.12% tetrahydrofuran; B, 5 mM ammonium acetate, pH 3.45, 58.5% acetonitrile, and 12.5% tetrahydrofuran). The gradient system comprised the following steps: 0−3.5 min 96% A, 12.5 min 83% A, 15.5 min 72% A, 20 min 60% A, 25 min 50% A, 27.5−33 min 100% B followed by reequilibration at 96% A. The flow rate was 0.31 mL/min. Electrospray tandem mass spectrometric analysis was carried out in electronegative mode. Conditions of analysis (in V) for catechins were as follows: focusing potential, −350; declustering potential, −21; electrode potential, −10; collision energy, −50; and collision energy exit potential, −20. Conditions of analysis (in V) for theaflavin, theaflavin monogallates, and theaflavin digallate, respectively, were as follows: focusing potential, −320, −340, and −350; declustering potential, −126, −121, and −101; electrode potential, −10.0, −9.0, and −7.0; collision energy, −44, −80, and −52; and collision energy exit potential, −10, −10, and −12. The ion spray voltage and temperature were −5000 V and 550 °C, respectively, for all polyphenols. Selective detection was achieved by multiple reaction monitoring of key tea polyphenols and their metabolites including the following species: theaflavin (*m*/*z* 563 > 125), theaflavin monogallates (715 > 125), theaflavin digallate (867 > 169), epicatechin (289 > 109), epigallocatechin (305 > 125), epicatechin-3-gallate (441 > 125), epigallogatechin-3-gallate (457 > 169), methyl epigallocatechin (319 > 125), epigallocatechin sulfate (385 > 137), and methyl epicatechin glucuronide (479 > 109).

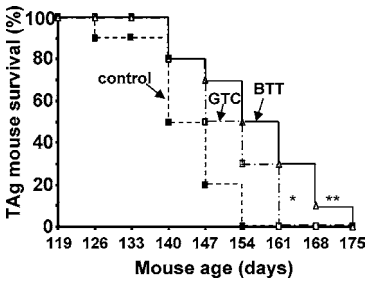

Figure 1. Effect of consumption of GTC (□-·-□) or BTT (△—△) (each 0.05% in the drinking water) on survival of TAg mice (study 1). Control animals received drinking water without tea preparation (■---■). Stars indicate that log rank analysis of the differences between mice on GTC or BTT vs control afforded $p < 0.05$ (*) or $p < 0.01$ (**), respectively.

Statistical Analyses. Statistical analyses were carried out by one-way analysis of variance with Tukey's pairwise comparison; analysis of survival (**Figure 1**) was performed by log rank test, both using SPSS for Windows (version 12.0.2).

RESULTS

Effect of GTE and BTT on Mammary Carcinogenesis. In the initial study, TAg mice (10 per group) received GTC or BTT (0.05%) with their drinking water from 4 weeks of age. Neither GTC nor BTT affected the intake of fluid or diet of mice as reflected by body weight of mice and measurement of remaining drinking water (results not shown). Administration of tea preparations increased TAg mouse survival slightly but significantly (**Figure 1**). The average age at death of animals was 144 ± 8 days for control mice, 151 ± 8 days for mice on GTC ($p = 0.27$ vs control), and 154 ± 16 days for mice on BTT ($p < 0.05$ vs control). The increase in survival was accompanied by slight, but not significant, decreases in number and volume of tumors per mouse in the intervention groups (data not shown). On pathological investigation, mice presented with intraduct hyperplasia, intraduct carcinoma, and invasive mammary carcinoma, which tended to be multicentric. Occasional intraduct papillomas were also present. These observations are consistent with the original description of the TAg mouse model (*21*). Histological inspection did not reveal significant differences in size or cytology of tumors between groups.

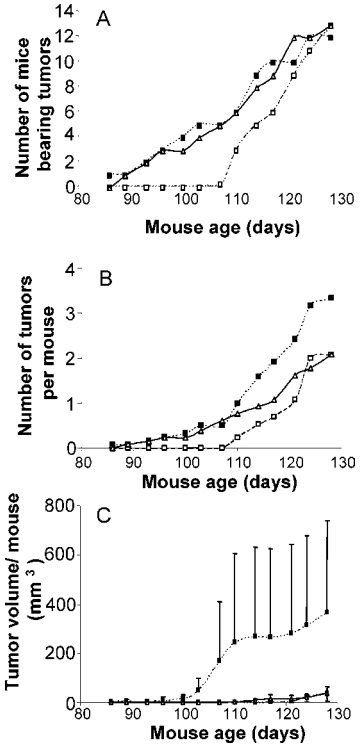

Figure 2. Effect of GTC (□) and BTT (△) (each 0.05% in the drinking water) on mammary tumor development in TAg mice as demonstrated by (**A**) number of mice with palpable tumors, (**B**) number of tumors per mouse, and (**C**) tumor volume per mouse (study 1). Control animals received drinking water without tea (■). Animals (14 per group) received tea preparations from weeks 4 to 19, after which they were killed. For the sake of clarity of representation, error bars have been omitted from **B**, where they were between 28 (BTT, day 89) and 98% of the mean (GTC, day 124). In **C**, tumor volumes in the intervention groups on day 100 and beyond day 110 were significantly lower than those in the controls ($P < 0.05$).

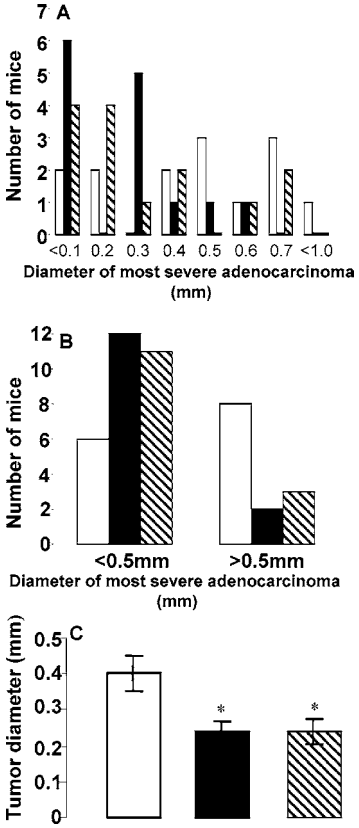

Figure 3. Effect of GTC (solid bars) and BTT (hatched bars) (each 0.05% in the drinking water) on tumor size in TAg mice (study 3), as reflected by size of largest tumor per mouse (**A**), number of mice with the size of the largest tumor either below or above 0.5 mm diameter (**B**), and mean tumor diameter (**C**). Open bars denote control animals. The star indicates that the difference between intervention and controls was significant ($P \leq 0.01$).

When this experiment was repeated (study 2), results were broadly similar to those described above. In study 2, an additional group of 16 mice received tea preparations at 0.01% in their drinking water. This intervention failed to affect mammary carcinogenesis. In study 3, which was terminated when mice were 19 weeks of age, intervention with either tea preparation impeded mammary carcinogenesis as demonstrated by the time of appearance of palpable tumors (**Figure 2A**), tumor number (**Figure 2B**), and tumor volume (**Figure 2C**). However, only in the latter case was the difference between intervention and control groups (in the late stages of tumor development) significant. Weights of tumor-bearing pelts of control and mice on GTC or BTT included in this study were 1.93 ± 1.85, 1.44 ± 0.53 ($p > 0.05$ vs control, $n = 16$ per group), and 1.18 ± 0.27 g ($p < 0.05$ vs control), respectively; thus, consumption of GTC and BTT decreased tumor load by 0.49 (25%) and 0.75 g (39%), respectively. When tissue sections from these mice were investigated microscopically, the size of the largest tumor lesion per mouse was significantly reduced in both GTC and BTT groups (42 and 40%, respectively, vis à vis controls; **Figure 3**). PCR analysis of tumor tissue showed that neither GTC nor BTT interfered with the expression of the SV40 transgene (result not shown).

Effect of GTE and BTT on M_1dG Adduct Levels. Exposure of MDA-MB-468 cells to GTC or BTT for 24 h caused a concentration-dependent decrease in M_1dG adduct levels (**Figure 4A**). Both tea preparations exhibited similar M_1dG adduct level-ameliorating potency. For comparative purposes, we also studied the effect of exposure to GTC and BTT on MDA-MB-468 cell proliferation. After exposure of cells to tea preparations for 48 h, the number of viable cells was significantly decreased by either GTC or BTT at 12.5 and 25 μg/mL, respectively (**Figure 5A**). This result suggests that the concentrations, at which tea preparations decrease M_1dG adduct levels, overlap with those that exert growth inhibition or induce apoptosis.

Mammary tumor tissue and blood leukocytes from TAg mice (study 2) were subjected to analysis of M_1dG adduct levels. Using tumors from control animals, the size of tumor was found to be without bearing on M_1dG content (result not shown). Adduct levels in normal breast tissue were too low to allow reproducible measurement. Intervention with either tea preparation significantly decreased M_1dG adduct levels in tumor tissue (**Figure 4B**). M_1dG adduct levels in tumors of mice on tea extracts (0.01 or 0.05%) were significantly reduced as compared to controls, for GTC by 50 ($p < 0.05$) and 78% ($p < 0.001$), respectively, and for BTT by 71 ($p < 0.001$) and 63% ($p < 0.05$), respectively. There was no difference in M_1dG levels in leukocytes between control mice and mice on either tea preparation (result not shown).

Effect of GTE and BTT on Tumor Tissue Proliferation. Mammary tumor tissue sections (study 3) stained for PCNA were inspected microscopically. All optical fields per experimental group ($n = 70$) were compared. The PCNA labeling index in tumors of control mice was $53 \pm 13\%$ (mean $\pm$ SD). In light of the typical cutoff point (30−45%) used to differentiate

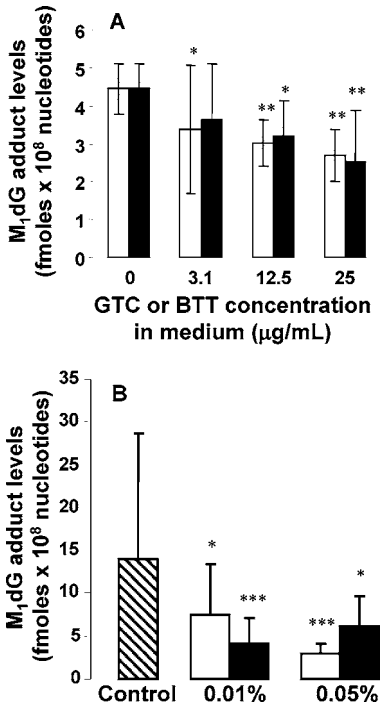

Figure 4. Effect of GTC (open bars) or BTT (closed bars) on levels of M_1dG in MDA-MB-468 breast carcinoma cells (**A**) and in mammary tumor tissue in TAg mice (study 2, **B**). Cells (in **A**) were incubated without (0 μg/mL) or with tea extracts (3.1–25 μg/mL) for 48 h, after which M_1dG levels were determined. In **B**, the concentration of GTC or BTT in the drinking water was 0.01 or 0.05%, and control animals received drinking water without tea extracts (hatched bar). Values are the means ± SD of six separate cellular incubations (**A**) or of groups of between 10 and 16 mice (**B**). The stars indicate that values were significantly different from the respective controls (*$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$).

between "low" and "high" PCNA indices, this value suggests that TAg mouse mammary tumors have a high proliferation rate. The PCNA labeling indices in mice on GTC or BTT were 50 ± 12 ($p = 0.27$ vs control) and 47 ± 10% ($p < 0.005$ vs control), respectively. Thus, consumption of BTT reduced proliferation in tumor significantly by 11%.

Effect of GTE and BTT on MDA-MB-468 Cell Survival and Cleaved Caspase 3 Levels in Mammary Tumors. In an orientation experiment, we explored whether intervention with GTC or BTT induces apoptosis. MDA-MB-468 cells exposed to GTC or BTT for 24 or 48 h were analyzed by flow cytometry using Annexin V FITC. Quantitative evaluation of fluororescence scattergrams (**Figure 5**) demonstrates that GTC at 12.5 and particularly potently at 25 μg/mL and BTT at 25 μg/mL decreased the number of viable cells and increased that of apoptotic cells. BTT significantly enhanced numbers of early apoptotic cells after incubation for 48 h (**Figure 5C**). In the case of GTC, only the late apoptotic cell fraction was significantly increased (**Figure 5F**). These alterations tentatively support the notions that both tea preparations can compromise the survival of breast cancer cells at least in vitro and that induction of apoptosis may contribute to this effect.

In mammary tissue of TAg mice (study 2), both tea preparations induced apoptosis as reflected by an increase in the level of cleaved caspase (**Figure 6**). GTC at 0.01% and BTT at 0.05% elevated protein levels by 67 and 38%, respectively.

Plasma and Tissue Levels of Catechins and Theaflavins in TAg mice. In four (of 10) mice on GTC (0.05%), plasma samples, which had been subjected to hydrolysis of conjugated metabolites, contained peaks consistent with epicatechin gallate

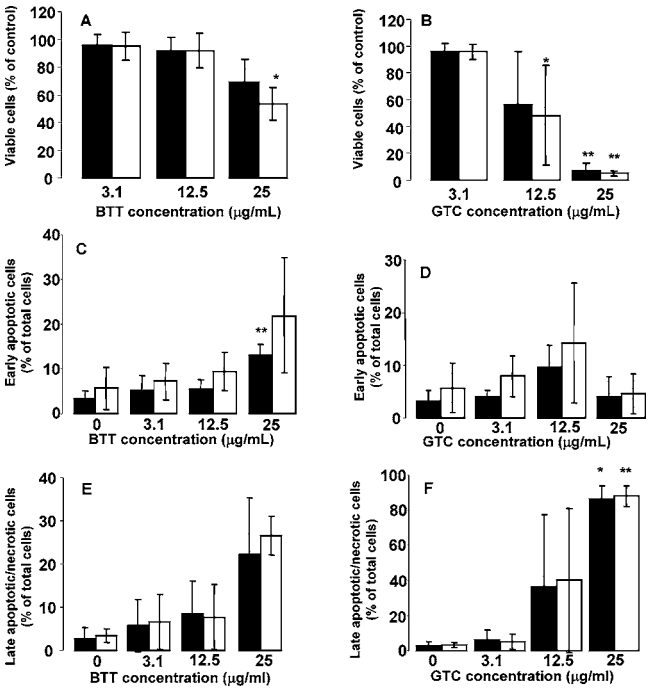

Figure 5. Effect of exposure to GTC or BTT on the number of viable and apoptotic MDA-MB-468 breast carcinoma cells. Bar diagrams show the effects of BTT (**A**, **C**, and **E**) or GTC (**B**, **D**, and **F**) at 3.1, 12.5, or 25 μg/mL on cell viability (**A**, **B**) and extent of early (**C**, **D**) or late (**E**, **F**) apoptosis after incubation for 24 (closed bars) or 48 h (open bars). Results were obtained by fluorescence-activated cell sorting using Annexin V FITC and propidium iodide. Values are expressed as percentage of total cells and are the means ± SD of three separate cellular incubations. The stars indicate that values were significantly different from the respective controls (*$p < 0.05$ and **$p \leq 0.01$).

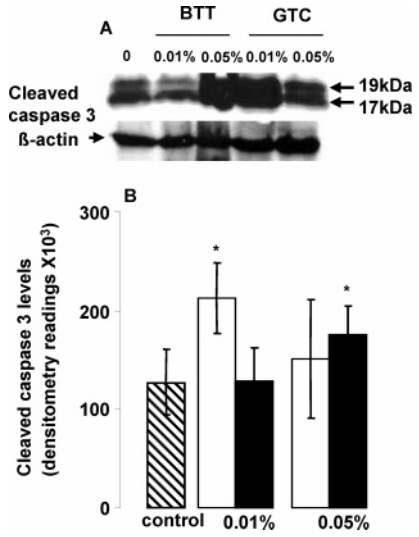

Figure 6. Effect of GTC (open bars) or BTT (closed bars) in the drinking water at 0.01 or 0.05% on expression of cleaved caspase 3 protein in mammary tumor tissue in TAg mice (study 3). Results are presented as a representative blot (**A**) or after quantitation by densitometry of cleaved caspase 3 bands in 10–16 individual mice (**B**). Values in **B** are expressed as arbitrary densitometry readings, and the control expression is shown by the hatched bar. The stars indicate that values were significantly different from the control ($p < 0.05$).

(**Figure 7A**,**B**). Unhydrolyzed plasma from two (of 10) mice afforded a peak consistent with methyl epicatechin sulfate (**Figure 7C**). Analysis of tumors from four (out of seven) mice

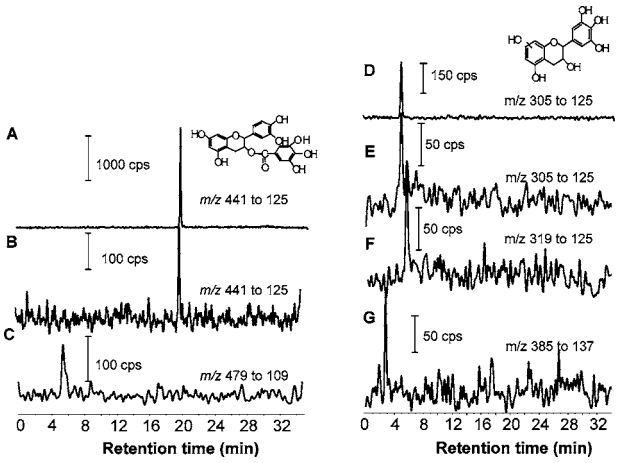

Figure 7. HPLC–tandem mass spectrometric analysis of solutions of authentic epicatechin gallate (**A**) or epigallocatechin (**D**) and of extracts of plasma (**B**, **C**) or tumor tissue (**E**–**G**) from TAg mice, which had received 0.05% GTC with their drinking water. Extracts were investigated by multiple reaction monitoring (with mass over charge ratios *m*/*z* in brackets) for the presence of epicatechin gallate (*m*/*z* 441 > 125; **A**, **B**), methyl epicatechin glucuronide (479 > 109; **C**), epigallocatechin (305 > 125; **D**, **E**), methyl epigallocatechin (319 > 125; **F**), and epigallocatechin sulfate (385 > 137; **G**). cps, counts per second. The plasma samples represented in **B** and **E** (but not the other extracts) were incubated with β-glucuronidase prior to extraction. Samples were prepared and analyzed as described in the Materials and Methods.

yielded epigallocatechin (after hydrolysis) and its methyl and sulfate metabolites (in unhydrolyzed samples; **Figure 7D−G**). Concentrations of epicatechin gallate in plasma were in the range of 72−320 nM (22−100 ng/mL), and epigallocatechin was present in tumor tissue at concentrations of 32−104 pmol/g (10−32 ng/g). The absence of available authentic standards of conjugated metabolites prevented their quantification. Other tea catechins or theaflavins or molecular species derived from them were not detected in either biomatrix, suggesting that levels were below the limits of detection for these polyphenols. The limits of detection for catechins and theaflavins were approximately 0.5 and 5 ng, respectively.

DISCUSSION

The results described above show for the first time that GTCs and BTTs delay mammary carcinogenesis in the TAg mouse model and that this delay is accompanied by an antioxidant effect in the target organ as reflected by levels of M_1dG adducts. Although green tea preparations have shown similar efficacy in mouse xenograft models (*14*, *35*−*37*), this is the first demonstration that theaflavins from black tea have comparable activity. Transgenic models have an advantage over xenograft models in chemoprevention studies, because the tumors develop in situ, are heterogeneous, and genetically resemble early stage carcinogenesis, whereas cell lines employed in xenograft models are derived from late stage disease and are homogeneous.

The delay of carcinogenesis was reflected by a slight prolongation of mouse survival in the case of BTT and by a significant decrease in volume and size of tumors in the mice exposed to either tea preparation. Pathological analysis failed to reveal cytological differences between malignancies in the intervention or control groups, consistent with the notion that tea extracts delayed mammary carcinogenesis in a subtle rather than a dramatic fashion. The extent of the delay was broadly similar for both GTC and BTT intervention groups, and it resembles that found previously in this model with 2-difluoromethylornithine and dehydroepiandrosterone (*38*). The moderate degree of efficacy observed here is consistent with the fact that only traces of catechins and catechin metabolites could be recovered from biomatrices of mice, which had received GTC. Polyphenols related to theaflavins were not detected in mice on BTT.

A variety of mechanisms have been suggested to contribute to the putative cancer chemopreventive properties of tea polyphenols including cell cycle dysregulation, enhanced apoptosis and inhibition of growth, angiogenesis, invasion, and metastasis (for a review, see ref *39*). It is probable that multiple mechanisms contribute to the overall chemopreventive efficacy of tea constituents in vivo, with the specific contribution of each mechanism to the overall activity being dependent upon the malignancy (*39*). A property, which has long been associated with cancer chemoprevention by tea polyphenols, is their antioxidative potency. The results described above demonstrate that intervention with either GTC or BTT significantly reduced M_1dG levels in vitro and in mammary neoplastic tissue, which reflect oxidative DNA changes. These findings are consistent with reports according to which green and black tea constituents possess similar antioxidant activities in rat liver homogenates in vitro (*40*) and in a biomimetic system, in which their effects on thiobarbituric acid-reactive substances and conjugated dienes generated during copper-induced low-density lipoprotein oxidation were assessed (*19*). The results presented here hint at the possibility that M_1dG levels may serve as a marker of efficacy for tea polyphenols, even though the lack of effect on leukocyte M_1dG levels is incompatible with their potential usefulness as a surrogate marker.

The delay in tumor development by the tea preparations described above may also be related to their ability to interfere with mammary tumor cell survival signaling. BTT and GTC increased tumor levels of cleaved caspase 3 protein indicating induction of apoptosis. In the chemoprevention paradigm, apoptosis can play a crucial role in eliminating preneoplastic and hyperproliferating cells from the organism; thus, its induction may contribute to the prevention of tumor progression. The results presented here are consistent with previous reports according to which green tea polyphenols induced apoptosis in mammary cells in vitro (*41*), increased levels of cleaved caspase 3 in mammary tumor tissue of BALB/c mice bearing the 4T1 murine mammary carcinoma (*42*), and increased apoptosis as demonstrated by TUNEL in a mouse xenograft model (*37*). However, in these papers, efficacy was seen at dietary doses that were 4−20 times higher than that employed by us. To our knowledge, black tea has hitherto not been shown to induce apoptosis in mammary tumor tissue. Previous investigations of apoptosis in this model using TUNEL demonstrated a biphasic apoptosis induction profile, in that the proportion of apoptotic cells was 1% of total cells in normal ductal epithelia, 13% in atypical mammary tissue and in mammary intraepithelial neoplasia, and 5% in invasive lesions (*25*). These differences were mirrored by expression levels of the proapoptotic protein Bax. The results presented here concerning levels of apoptosis in tumors of mice that received tea extracts do not allow any inference to be made as to whether intervention affected the biphasic apoptosis profile. It is just conceivable that the absence of a proper dose−response relationship for caspase 3 induction by intervention with GTC (**Figure 6**) may be related to changes in the level of apoptosis during tumor development in this model.

In the case of BTT, efficacy may also have been in part related to the slight but significant decrease in rate of tumor cell proliferation. Recently, intervention with green tea polyphenols or EGCG was shown to significantly reduce the proliferation index in xenograft tumors as reflected by PCNA staining (*37*). The discrepancy between this result and that described above, according to which GTC failed to affect proliferation in TAg mouse tumor tissue, may be related to the 20-fold difference in dose between the two studies. The dose of GTC and BTT shown here to delay mammary carcinogenesis (0.05%) corresponds to approximately three and 15 cups of green and black tea, respectively, per day, when the dose based on polyphenol content in tea extracts is extrapolated from mice to humans (70 kg) using the body surface area calculation described by Freireich et al. (*43*). While the content of theaflavins in black tea extracts is generally between approximately 2 and 6%, the black tea preparation used in the study described here consisted exclusively of theaflavins, which explains the high cuppage equivalent of the BTT dose. If further studies support beneficial health effects of theaflavins, it may be propitious to consider elevation of the theaflavin content in commercial black tea products, which can be achieved through selective processing techniques.

Only epicatechin gallate in plasma and epigallocatechin in tissue were detected after interventions with GTC. There was no evidence of the presence of EGCG after intervention with GTC or of theaflavins after BTT intake. The absence of measurable levels of theaflavins may be related to the 10-fold lower sensitivity of our detection system for these components as compared to the detection limit for catechins and/or their limited bioavailability (*20*). Little is known about the fate of theaflavins in biological systems. It is conceivable that they are present as yet unidentified breakdown products or metabolites. The target organ concentrations of catechins measured in studies such as the one described here are 2−3 orders of magnitude lower than the concentrations required to elicit a pharmacological response in cells in vitro. Nevertheless, one cannot discount the possibility that lifetime exposure to low levels of tea polyphenols may be sufficient for causation of efficacy.

The TAg mouse is a model of an insidious type of human estrogen receptor-negative breast cancer. The finding described here, that tea preparations delay the development of mammary carcinogenesis in the TAg mouse, might have clinical implications. There is currently no generally accepted clinical management option for women presenting with DCIS. The results described here intimate that it is worthwhile to explore the ability of tea extracts to delay progression from DCIS to full-blown mammary carcinoma in humans. The low toxicity, low cost, and ease of consumption of tea render such a strategy attractive.

ABBREVIATIONS USED

BTT, black tea theaflavins; DCIS, ductal carcinoma in situ; EGCG, epigallocatechin-3′-gallate; GTC, green tea catechins; M_1dG, 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-α]-purin-10(3*H*)one; PCNA, proliferating cell nuclear antigen; TAg mouse, C3(1) SV40 *T*,*t* antigen transgenic multiple mammary adenocarcinoma mouse; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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