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Polyphenols in brewed green tea inhibit prostate tumor xenograft growth by localizing to the tumor and decreasing oxidative stress and angiogenesis☆

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Abstract

It has been demonstrated in various animal models that the oral administration of green tea (GT) extracts in drinking water can inhibit tumor growth, but the effects of brewed GT on factors promoting tumor growth, including oxidant damage of DNA and protein, angiogenesis and DNA methylation, have not been tested in an animal model. To explore these potential mechanisms, brewed GT was administered instead of drinking water to male severe combined immunodeficiency (SCID) mice with androgen-dependent human LAPC4 prostate cancer cell subcutaneous xenografts. Tumor volume was decreased significantly in mice consuming GT, and tumor size was significantly correlated with GT polyphenol (GTP) content in tumor tissue. There was a significant reduction in hypoxia-inducible factor 1-alpha and vascular endothelial growth factor protein expression. GT consumption significantly reduced oxidative DNA and protein damage in tumor tissue as determined by 8-hydroxydeoxyguanosine/deoxyguanosine ratio and protein carbonyl assay, respectively. Methylation is known to inhibit antioxidative enzymes such as glutathione *S*-transferase pi to permit reactive oxygen species promotion of tumor growth. GT inhibited tumor 5-cytosine DNA methyltransferase 1 mRNA and protein expression significantly, which may contribute to the inhibition of tumor growth by reactivation of antioxidative enzymes. This study advances our understanding of tumor growth inhibition by brewed GT in an animal model by demonstrating tissue localization of GTPs in correlation with inhibition of tumor growth. Our results suggest that the inhibition of tumor growth is due to GTP-mediated inhibition of oxidative stress and angiogenesis in the LAPC4 xenograft prostate tumor in SCID mice.

Keywords: Green tea; LAPC4 prostate xenograft tumor; Oxidation; Angiogenesis; Methyltransferases; Macrophage invasion

1. Introduction

Numerous studies in cell culture and in animal models demonstrate that either green tea extract (GTE) or purified (−)-epigallocatechin gallate (EGCG) [1–3] can inhibit tumor cell proliferation and xenograft tumor growth. Meta-analyses of epidemiological studies demonstrate a small but significant reduction in the risk of breast, lung and stomach cancer in individuals consuming brewed green tea [4–6]. Consumption of 600 mg/day of a GTE by men with high-grade prostate intraepithelial neoplasia (PIN) significantly delayed the progression of PIN to prostate cancer (CaP) [7]. The active phytochemicals in GT are the green tea polyphenols (GTPs), also known as flavan-3-ols, including (−)-epigallocatechin (EGC), EGCG, (−)-epicatechin (EC) and (−)-epicatechin-3-gallate (ECG). While EGCG is the most active and abundant polyphenol, we have previously demonstrated that natural products exert their beneficial effects based on the sum of the multiple mixed components [8]. GTPs can exhibit antioxidant as well as prooxidant activity in cell culture. The antioxidant activity of GTPs derives from their direct radical scavenging activity via electron transfer from hydroxyl groups in the polyphenol ring and indirectly through activation of the nuclear antioxidant response element via the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor [9,10]. Prooxidant activity *in vitro* results from the autooxidation and dimerization of EGCG and EGC to form homo- and heterodimers in an alkaline environment with concurrent formation of hydrogen peroxide [11]. Mitochondrial respiratory chain metabolism and a number of enzymatic reactions including those involving NAD(P)H oxidases, xanthine oxidase, myeloperoxidase, cyclooxygenase and lipoxygenase can serve as endogenous sources of reactive oxygen species (ROS) [12,13]. Macrophage infiltration in CaP has been identified universally in prostatectomy tissue [14]. In animal models, macrophage infiltration has been demonstrated in orthotopically transplanted human prostate tumors [13]. Inflammatory macrophages release ROS, cytokines, chemokines and prostaglandins which can lead to tissue remodeling and angiogenesis [14,15].

Prostate tumors are characterized by a down-regulation of key antioxidant enzymes such as glutathione *S*-transferase pi (GSTp1)

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and manganese superoxide dismutase through epigenetic silencing of CpG island hypermethylation [16–18], suggesting that tumor cell proliferation is dependent on a minimal level of ROS.

EGCG has been shown to inhibit 5-cytosine DNA methyltransferase 1 (DNMT1) [19], leading to demethylation of the CpG islands in the promoters and the reactivation of methylation-silenced genes such as p16INK4a, retinoic acid receptor beta, O6-methylguanine methyltransferase, human mutL homolog 1 and GSTp1 [20]. Since CaP is commonly associated with hypermethylation and silencing of GSTp1, it is possible that GT at a cellular level may reactivate GSTp1 [21,22], resulting in tumor growth inhibition by reducing the concentration of ROS needed to maintain tumor growth.

Most prior investigations of the mechanisms underlying the cancer preventive activities of GT have utilized either EGCG alone or decaffeinated GTEs highly enriched in EGCG [23]. The objective of the present study was to administer brewed GT in drinking water to male severe combined immunodeficiency (SCID) mice bearing a human prostate cancer xenograft (LAPC4) to mimic human tea consumption and to determine the effects on tumor growth, intratumoral GTPs, macrophage infiltration, oxidant stress, angiogenesis, and damage to tumor DNA and protein.

2. Methods and materials

2.1. Green tea intervention

Green tea was brewed every 3 days by steeping one tea bag in 250 ml of boiling water (pH 3) for 5 min. Tea bags (Authentic Green Tea) were generously provided by Celestial Seasonings (Boulder, CO, USA). The stability of GTP was tested and was stable for 3 days. The GTP composition of the brewed green tea in mg/L was as follows: EGC 202±11, EGCG 397±28, EC 52±5, ECG 62±5 and catechin 8±2.

2.2. Animal studies

All procedures carried out in mice were approved by the UCLA Animal Research Committee. Male SCID mice (Charles River Laboratories) were bred in a pathogen-free colony [24], where they were housed in groups of five per cage and fed a sterilized AIN-93G diet (DYETS, Inc., Bethlehem, PA, USA) and water. The brewed GT was provided in the same manner as drinking water. Androgen-dependent LAPC4 prostate cancer cells mixed in 0.1 ml of matrigel (5×10^5 LAPC4 cells per animal; gift from Dr. Charles Sawyers) were implanted subcutaneously into the flank of 5-week-old SCID mice. A small preliminary experiment (experiment 1) was performed to determine oxidative DNA damage in LAPC4 xenograft tumors in mice drinking water *ad libitum*. Control mice were left intact, and intervention mice were surgically castrated when tumors were palpable (about 4 weeks after inoculation) ($n=5$). When tumors reached a volume of 1 cm^3, mice were sacrificed, and tumors and the prostate gland (all four lobes) were removed and frozen for later oxidative DNA damage determination. In experiment 2, to test the chemopreventive effect of brewed green tea, GT was administered in drinking water for 13 weeks ($n=5$ per group) orally 7 days per week starting 2 weeks after LAPC4 tumor cell inoculation. Tumor size was measured with calipers three times a week starting at day 7. Tumor volume was calculated using the formula length×width×height×0.5236 [25]. Mice in the control group were sacrificed after 8 weeks postinoculation and mice in the tea group 13 weeks after the inoculation to obtain serum samples, liver, lung, kidney and tumor tissue. Tumors were excised and rinsed in cold phosphate-buffered saline. A 2-mm slice was cut in longitudinal direction from the center of the tumor and transferred into a tissue cassette, fixed in 10% neutral buffered formalin for 12 h and transferred to 70% alcohol for immunohistochemistry [26]. The remaining tumor tissue was cut into six to eight sections, carefully labeled according to the location in the tumor (outside, inside, center) and immediately frozen in liquid nitrogen together with other tissues for further analyses. The GT intervention study using brewed GT was performed twice with $n=5$ per group in each experiment. The outcomes were very similar in both experiments.

2.3. Immunohistochemistry of microvessel density and macrophage presence

Paraffin-embedded sections were cut at 4-μm thickness, and paraffin was removed with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Heat-induced antigen retrieval, 95°C for 25 min (microvessel density), and proteolytic-induced epitope retrieval were carried out with proteinase K (Dako, S3020, Carpinteria, CA, USA) at 37°C for 10 min (F4/80). To quantify microvessel density, goat polyclonal Pecam-1 (CD31) (Santa Cruz, sc-1506, Santa Cruz, CA, USA) primary antibody at 1:200 dilution for 1 h and F4/80 primary antibody (Serotec MCA497b, Raleigh, NC, USA) at 1:50 dilution overnight at 4°C followed by 30 min with secondary polyclonal rabbit anti-goat immunoglobulins/biotinylated (Dako, E0466, Carpinteria, CA, USA) at 1:200 were used. The signal was detected using the mouse DAKO horseradish peroxidase EnVision kit (DAKO) and anti-rabbit HRP polymer and visualized with the diaminobenzidine reaction. The sections were counterstained with hematoxylin. Number of vessels was manually counted in 20 fields at 400× magnification using the Ariol SL-50 automated slide scanner (Applied Imaging, San Jose, CA, USA) at the Translational Pathology Core Laboratory, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA. The presence of F4/80-stained macrophages was assessed by semiquantitative scoring of staining intensity of the cells grouped into five grades: 0, lack of expression; 1+, low expression in less than 10% of cells; 2+, low to moderate expression in 11% to 30%; 3+, moderate to strong staining in 31% to 50%; and 4+, strong expression in 50% or more.

2.4. Western blot analysis of protein expression in mouse tumors

Fifty micrograms of protein was used for the analysis of hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (VEGF) and DNMT1. Protein was separated on a 4%–20% Tris-HCl gel. Separated proteins were electrotransferred to nitrocellulose membranes and blocked in Tris-buffered saline with 0.1% Tween 20 and 5% nonfat milk for 1 h at room temperature. Membranes were incubated with primary antibody against HIF-1α (sc-10790), VEGF (sc-152) or DNMT1 (sc-20701, Santa Cruz) at a dilution of 1:100 overnight at 4°C. Protein was visualized and analyzed using a ChemiDoc XRS (Bio-Rad Laboratories) chemiluminescent detection and imaging system. After stripping the membrane, monoclonal antibody to β-actin or GAPDH was applied as loading control.

2.5. Oxidative DNA and protein damage

DNA was extracted from 80–100 mg of tumor tissue by the phenol/chloroform/i-soamyl alcohol extraction method described by Hofer et al. [27]. One hundred micrograms of DNA was digested according to Huang et al. [28], and the concentrations of 8-hydroxydeoxyguanosine (8-oxo-dG) and total deoxyguanosine (dG) were determined by high-performance liquid chromatography (HPLC) using Coulochem II electrochemical detection (ESA, Chelmsford, MA, USA) as described previously [29]. Protein oxidation was determined by concentration of protein carbonyl using the Cayman protein carbonyl assay kit (Cayman Chemical Company, Ann Harbor, MI, USA) according to manufacturer's instructions.

2.6. Tea polyphenol analysis

One hundred fifty milligrams of the tumor tissue was homogenized in 200 μl of 2% ascorbic acid solution on ice and transferred to 2-ml tubes. The homogenate was incubated with 1000 U of β-glucuronidase (G7896, Sigma Chemicals, St. Louis, MO, USA) and 40 U of sulfatase (S-9754, Sigma Chemicals) buffered in 300 μl of 0.5 M phosphate buffer (pH 5.0) at 37°C for 45 min to digest the conjugated forms into their free forms. After incubation, 4× extracts with 1 ml of ethyl acetate were combined with 20 μl of 2% ascorbic acid in methanol and dried in vacuum and reconstituted for HPLC CoulArray detection (ESA, Chelmsford, MA, USA) [24]. The compounds were separated on an Alltima C18 reverse-phase column (3 μm, 53×7 mm) (Alltech Associates Inc., Deerfield, IL, USA) as described by Henning et al. [24]. The eight channels of the CoulArray detector (ESA, Chelmsford, MA, USA) were sequentially set at −60-, 20-, 100-, 180-, 260-, 340-, 420- and 500-mV potentials.

2.7. Quantitative real-time polymerase chain reaction (PCR) of DNMT1

PCR primers and fluorogenic probes were provided by TaqMan Gene Expression Assay kit (ID: Hs00154749_m1 for DNMT1) (Applied Biosystems, Foster City, CA, USA). The final volume of 20-μl PCR mixture contained 2 μl of cDNA template, 1 μl of 20× primer and probe mixture, 10 μl of 2×TaqMan Universal PCR MasterMix (Applied Biosystems) and 7 μl of nuclease-free water. PCR amplification was performed by a 7900HT Fast Real-Time System (Applied Biosystems) with the following thermal cycling conditions: 50°C for 2 min followed by 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was in triplicate. In addition, each run included no-template negative controls. The $2^{-(\Delta\Delta Ct)}$ method was used to normalize the expression of DNMT1 in each sample to GAPDH expression and to compare to the average ΔCt value.

2.8. Statistical methods

SPSS (Version 17.0, Chicago, IL, USA) and Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, USA) were used for statistical analyses. Mean value, median and standard deviation (S.D.) were calculated using descriptive statistics. Comparison of means was performed by two independent-samples *t* test. Differences were considered significant if $P<.05$.

3. Results

3.1. Tumor volume, tissue GTP content and GTP metabolism

GT prepared from commercially available tea bags contained a mixture of GTPs as found in GT leaves. To evaluate whether brewed GT, as consumed in the general population, inhibits prostate tumor growth in SCID mice implanted with LAPC4 human androgen-dependent prostate tumor cells, we replaced drinking water with brewed GT. There was no difference in body weight between GT-consuming and control mice (Fig. 1A). After 5 weeks, tumor volume was inhibited significantly in GT-consuming mice. After 11 weeks, tumor growth reached a plateau (Fig. 1B).

EGCG, 4″-*O*-methyl-EGCG (4″-MeEGCG), EGC, 4′-*O*-methyl-EGC (4′-MeEGC) and EC were found in the tumor tissue of mice consuming GT (Fig. 2). The identity of the 4″-MeEGCG and 4′-MeEGC standards was confirmed by liquid chromatography/tandem mass spectrometry analysis, and excellent correlation with the HPLC analysis with CoulArray electrochemical detection was established. EC, EGC and 4′-MeEGC were found mostly in the conjugated (glucuronidated+sulfated) form (86%±4%, 75%±4% and 83%±4% of total, respectively). EGCG and 4″-MeEGCG were present in the free form at 68%±5% and 50%±5% of total, respectively. The total concentration of GTPs and methyl metabolites was significantly correlated to tumor volume (r=0.94, P<.007). The administration of brewed GT inhibited the gene and protein expression of DNMT1 significantly (55% and 40%, respectively; Fig. 3A,B).

3.2. GT effects on oxidative DNA and protein damage and macrophage presence

In a preliminary study (experiment 1), we compared the level of oxidative DNA damage in xenograft tumor tissue to prostate gland tissue including prostate tissues from intact mice and from castrated mice (Fig. 4A). In this experiment, we established that the ratio of 8-oxo-dG to dG was significantly increased in LAPC4 xenograft tumor tissue compared to normal prostate tissue. There was no significant difference in oxidative DNA damage in normal tissues from castrated mice compared to intact mice. In xenograft tumors from SCID mice consuming brewed GT, the ratio of 8-oxo-dG to dG was significantly decreased by 60% compared to tumors from control tumor-bearing mice consuming plain water (Fig. 4B). In addition, we demonstrated that the concentration of carbonyl protein as an indicator of oxidized protein was significantly decreased by 60% in GT-drinking mice compared to the tumor-bearing controls consuming water alone (Fig. 4C).

F4/80 staining of tumor sections also indicated the presence of macrophages. Macrophages were distributed throughout the tumor section in an irregular manner, mainly accumulating in areas bordering the invasive zone. Fig. 6 (A) demonstrates an example of a tumor from a tea-treated mouse with reduced macrophage

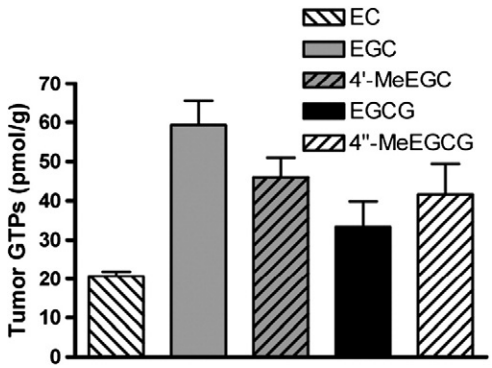

Fig. 2. Concentration of total (free+conjugated) GTPs and their methyl metabolites in tumor tissue of mice treated with green tea (means±S.D., n=5, *P≤.05).

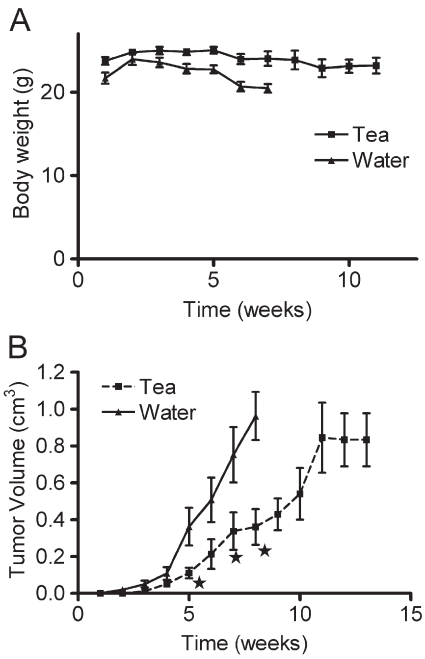

Fig. 1. Body weight (A) and tumor volume (B) of SCID mice inoculated with LAPC4 prostate tumor cells and treated with brewed green tea or water (means±S.D., n=5, *P≤.05).

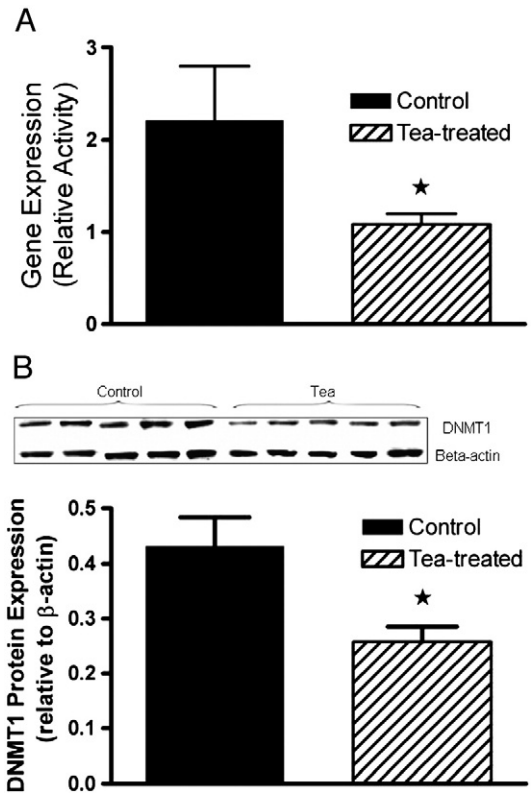

Fig. 3. (A) Gene expression of DNMT1 and (B) protein expression of DNMT1 in tumor tissue of mice treated with tea or water determined by quantitative real-time PCR (means±S.D., n=5, *P≤.05).

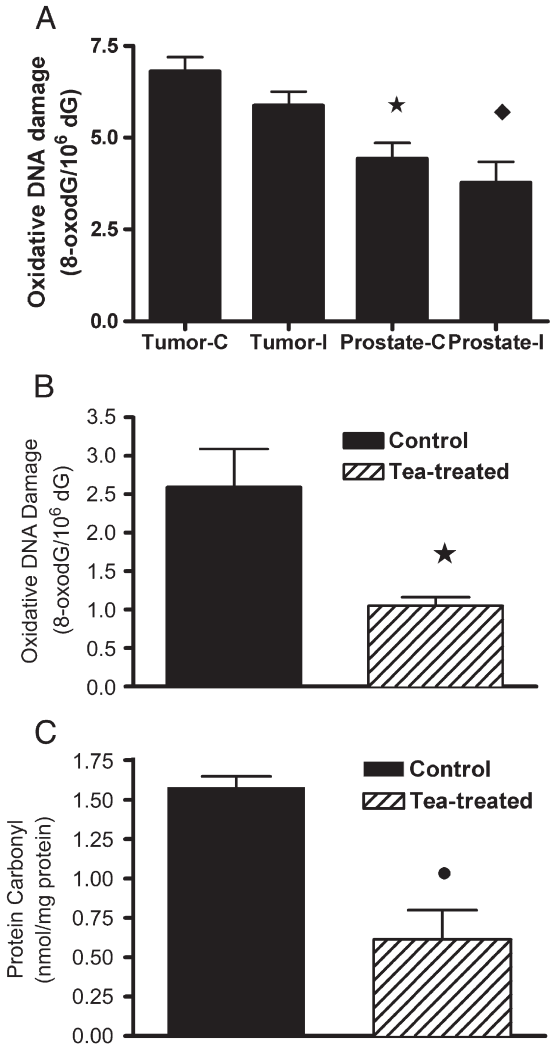

Fig. 4. Oxidative DNA damage (A) in xenograft LAPC4 tumor or normal prostate tissue in intact (I) or castrated (C) mice; significant difference between tumor and prostate in intact (♦) and castrated mice (*). (B) Oxidative damage in tumor tissue and (C) oxidative protein damage in tumor tissue of mice treated with green tea or water (means±S.D., $n=5$, $^*P \leq .05$).

infiltration. However, overall, there was no significant difference in F4/80-positive macrophage marker staining demonstrated in tumors from tea-treated and control mice.

3.3. GT effects on markers of angiogenesis

In addition to the antioxidant effects, we determined the effect of consumption of brewed GT on markers of angiogenesis. Protein expression of HIF-1α and VEGF in tumor tissue was significantly decreased by 60% and 35% in mice drinking brewed GT (Fig. 5A, B). Vessel density showed a nearly significant trend to decrease by 42% from 26±8.9 to 15.2±2.5 microvessels per field ($P=.058$) (Fig. 5C).

4. Discussion

Polyphenolic botanical extracts such as GT exert their effects on tumor growth through multiple concurrent direct and indirect mechanisms captured under the term of antioxidant effects [30]. In this study, we demonstrated that brewed GT inhibited markers of oxidative damage in the prostate cancer xenograft tumor tissue. In addition, many effects are beyond those that can be characterized as simply antioxidant effects. Pathways involved in antioxidant defense, redox status, inflammation and methylation are closely interrelated and interact with the multistep process of carcinogenesis [31].

Angiogenesis is a component of the inflammatory pathway involved in tumor promotion, and tumors cannot grow without stimulating new blood vessel formation [32]. New vessel formation is triggered by hypoxia via stimulation of VEGF and the HIF-1α pathways [33]. We demonstrated a significant decrease in tumor tissue angiogenesis-related HIF-1α and VEGF protein expression and an almost significant trend ($P=.058$) of decrease in microvessel density. A possible mechanism of HIF-1α inhibition has been described in PC-3 prostate cancer cells where 20–40 μmol of EGCG inhibited the prolyl hydroxylation of HIF-1α, thus preventing the interaction of HIF-1α with von Hippel–Lindau protein necessary for

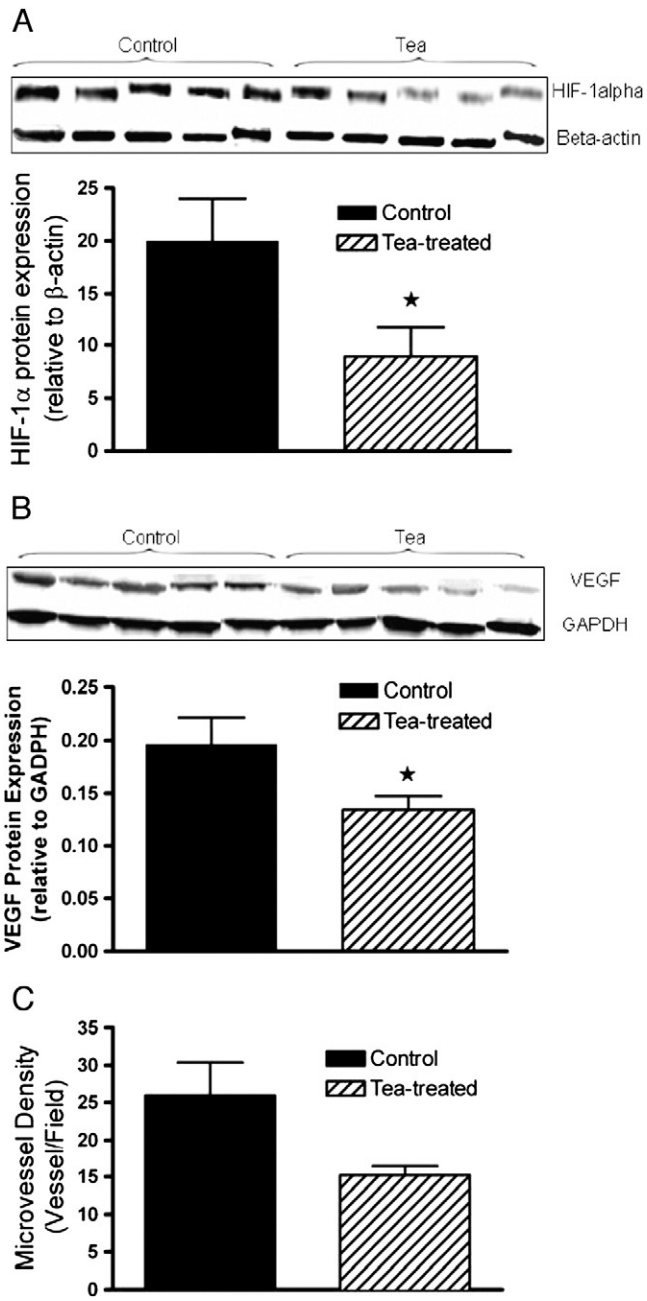

Fig. 5. Protein expression of (A) HIF-1α and (B) VEGF in tumor tissue of mice treated with green tea or water by Western blot and (C) microvessel density by immunohistochemistry (means±S.D., $n=5$, $^*P \leq .05$).

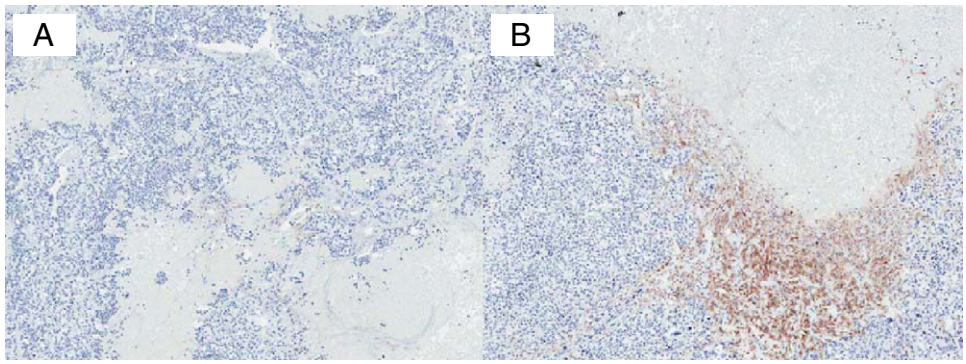

Fig. 6. Macrophage presence in tumor tissue of mice treated with green tea (A) or water (B) by F4/80 immunohistochemistry staining (×50 magnification).

angiogenesis stimulation [34]. The inhibition of VEGF protein expression has been demonstrated before by other investigators in a xenograft prostate cancer model and TRAMP mice treated with a GTE enriched for EGCG [35,36]. However, our results are unique to demonstrate the inhibition of these angiogenesis factors in mice consuming brewed GT instead of drinking water.

The xenograft model is not a perfect representation of human prostate cancer. In SCID mice, inflammatory processes, driven by mechanisms inherent to the host-tumor immune reaction, may be preserved but may be nonresponsive to GT treatment. Although the GT intervention did not affect macrophage density in the present study, this does not rule out an inhibitory effect in human prostate tissue where the variety of cells infiltrating may be different, including different subtypes of macrophages [37]. Tumor-associated macrophages also have the capacity to generate ROS through NADPH oxidase activity and stimulate angiogenesis [38,39]. Decreasing the macrophage infiltration or modulating the tumor-associated macrophage subpopulations could be another potential mechanism through which GT could lower oxidative stress and inhibit angiogenesis in tumor tissues indirectly. This area clearly deserves more investigation, but the design of experiments using the xenograft model will be critical in advancing our understanding of intratumoral immune effects of GT.

The antioxidant activity of GTPs in human and animal studies has been reviewed extensively [40,41], and there is evidence from human intervention studies that brewed GT and GT extract supplements exert an antioxidant activity [24,42,43]. In the present study, we observed a significant inhibition of oxidative DNA and protein damage in tumor tissue from mice exposed to brewed GT. While it is possible that some of these effects were the result of a direct chemical antioxidant activity of GTPs via electron transfer and radical scavenging of metal ions [44], the low plasma and tissue concentrations of GTPs and the presence of many other stable antioxidant defense systems in the tissues make this unlikely [45]. In our view, it is more likely that GTPs act through up-regulation of antioxidant defense mechanisms via activation of the Nrf2 axis [10] and reactivation of silenced antioxidant enzyme genes through a decrease in DNA methylation [20]. Stage-specific alterations of DNA methyltransferase expression, gene specific DNA hypermethylation and also global DNA hypomethylation have been defined in different stages of prostate carcinogenesis in transgenic (TRAMP) mice [46]. A recently published work by Pandey et al. showed that treatment with GTPs *in vitro* caused promoter demethylation and chromatin remodeling leading to expression of GSTP1 in human prostate cancer cells, which had been suppressed [47].

Our results demonstrate for the first time *in vivo* that the consumption of brewed GT is associated with significant inhibition of gene and protein expression of DNMT1 in xenograft tumors. A study by Morey Kinney et al. in transgenic TRAMP mice did not demonstrate a decrease in DNA methylation [48], but also failed to demonstrate inhibition of tumor progression in TRAMP mice. Therefore, there is no conflict in the findings from the above transgenic study and our findings. Nonetheless, further studies are needed to investigate whether the inhibition of DNMT1 is sufficient to reverse gene-specific DNA hypermethylation in human prostate tissues following GT administration.

A related effect of decreasing the methylation of GTPs is the increase in antiproliferative activity. In previous work, we established that methylation of EGCG was associated with a decrease of antiproliferative activity, decreased inhibition of nuclear factor-κB activation and decreased apoptosis [49]. In the present study, 56%±6% of EGCG and 45%±5% of EGC were found in methylated form, which may have significantly limited their potential to inhibit tumor growth. Similar amounts of methyl-EGCG were found in prostate tissue as part of an ongoing phase II human intervention study in men consuming green tea prior to prostatectomy [49]. We are currently investigating the possibility that combinations of agents that inhibit methylation might enhance the chemopreventive activity of green tea in prostate carcinogenesis.

In summary, our study has shed light on potential mechanisms through which GTPs from brewed green tea as opposed to purified or concentrated EGCG might affect prostate carcinogenesis. In this regard, our research attempts to recapitulate in animal models and human studies the effects observed in epidemiological studies. It is hoped that this translational research will lead to new approaches to prostate cancer prevention.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2011.10.007.

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