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Epigallocatechin-3-Gallate Suppresses Early Stage, but not Late Stage Prostate Cancer in TRAMP Mice: Mechanisms of Action

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BACKGROUND. Prostate cancer (PCa) is the second leading cause of cancer-related death in men in the United States. Many men have implemented purported chemopreventive agents into their daily diet in an attempt to delay the early onset of a PCa. Green tea polyphenols, one such agent, has been shown to be chemopreventive in skin, breast, and prostate cancers. We hypothesized that Epigallocatechin-3-Gallate (EGCG), the major polyphenol found in green tea, will exert its chemopreventive effect in the prostate via regulation of sex steroid receptor, growth factor-signaling, and inflammatory pathways.

METHODS. Five-week-old male TRAMP (Transgenic Adenocarcinoma Mouse Prostate) offspring were fed AIN-76A diet and 0.06% EGCG in tap water. Animals were sacrificed at 28 weeks of age and the entire prostates were scored histopathologically. In addition, animals were sacrificed at 12 weeks of age and ventral (VP) and dorsolateral (DLP) prostates were removed for histopathological evaluation and immunoblot analyses or ELISA.

RESULTS. EGCG, inhibited early but not late stage PCa in the current study. In the VP, EGCG significantly reduced cell proliferation, induced apoptosis, and decreased androgen receptor (AR), insulin-like growth factor-1 (IGF-1), IGF-1 receptor (IGF-1R), phospho-extracellular signal-regulated kinases 1 and 2 (phospho-ERKs 1 and 2), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS).

CONCLUSIONS. The attenuation of the AR, the down-regulation of potent growth factor IGF-1, modulation of inflammation biomarkers, and decrease in the MAPK signaling may contribute to the reduction in cell proliferation and induction of apoptosis and hence provide a biochemical basis for EGCG suppressing PCa without toxicity. *Prostate 67: 1576–1589, 2007.*

KEY WORDS: **EGCG; TRAMP; AR; IGF-1; prostate cancer; chemoprevention**

INTRODUCTION

Prostate cancer (PCa) is a disease that is responsible for approximately 40,000 deaths per year and is the second-leading cause of cancer-related death among men in the United States [1]. PCa starts to develop early in life with pre-neoplastic lesions appearing as early as 40–50 years of age in men [2,3]. High-grade prostatic intraepithelial neoplasia (HG-PIN) is considered the precursor to prostate adenocarcinoma [4]. HG-PIN has a high predictive value as a

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marker for prostate adenocarcinoma, and its identification warrants biopsy for invasive carcinoma.

Green tea polyphenols (GTPs) have been shown to be effective in preventing pre-malignant lesions before PCa develops in humans [5]. In addition, Gupta et al. and others have demonstrated the ability of green tea polyphenols (GTPs) to suppress PCa in the Transgenic Adenocarcinoma Mouse Prostate (TRAMP) model [6–8]. Studies have linked the low incidence of PCa in Chinese populations to the consumption of green tea. [9,10]. However, other studies have failed to find an association between green tea consumption and the risk of PCa [11,12]. Despite opposing epidemiological data, many men have implemented purported chemopreventive agents, such as green tea extracts, into their daily diet in an effort to delay the progression of PCa.

Green tea is comprised of a number of polyphenolic compounds known as catechins including epigallocatechin-3-gallate (EGCG), epicatechin (EC), epigallocatechin (EGC), and epicatechin-3-gallate (ECG). EGCG, the most abundant GTP in green tea, accounts for 50–80% of the tea catechins. Furthermore, EGCG has been credited with many of the health benefits of green tea. Yet, it remains unclear whether these benefits are due to EGCG alone or if an additive or synergistic effect exists in green tea due to the combination of GTPs. Studies using GTPs in PCa have focused on an array of signaling pathways responsible for maintaining a balance between proliferation and apoptosis. The insulin-like growth factor-1 (IGF-1) ligand, and its corresponding binding proteins and receptor are important in normal prostate growth and development as well as PCa initiation and progression. The IGF-1 signaling pathway can activate the Ras/MAPK and PI3K/Akt pathways. High circulating levels of IGF-1 in the blood have been associated with an increased risk of PCa in humans [13,14]. In the TRAMP model, Adhami et al. [15] showed a decrease in IGF-1 and an increase in IGF-binding protein 3 (IGF-BP3) levels in the blood serum of GTP-treated animals. In addition, phospho-extracellular signal-regulated kinases 1 and 2 (phospho-ERKs 1 and 2), components of the MAPK pathway responsible for cell proliferation, differentiation, and death, were down-regulated by GTP in TRAMP mice [15].

In comparison, ECGG as a single agent can modulate the production and biological actions of androgens and other hormones [16]. The androgen receptor (AR) plays a dominate role in the molecular endocrinology of PCa. AR participates in tumor progression by activating and/or up-regulating receptor activity, point mutations, and ligand-independent activation by growth factor signaling pathways (i.e., IGF-1) [17]. Using LNCaP cells PCa cells, Ren et al. [18] described the down-regulation of the AR expression with EGCG. Evidence shows that 85 mg EGCG/kg BW can regulate the endocrine system by lowering testosterone and estradiol concentrations in the blood serum of male Zuker rats [19]. EGCG also modulates inflammation by inhibiting MMP-2 activation [20] in TRAMP-C1 cells and acts as an anti-oxidant by alleviating oxidative and nitrosative injuries in TRAMP mice [21].

It is of fundamental importance to determine if the biological responses caused by green tea are caused solely by EGCG prior to utilizing EGCG as a single agent in clinical trials or taking EGCG supplements. In the current study, we investigated the potential of EGCG, as a single agent, to protect against the progression of PCa using the TRAMP model, an animal that closely mimics PCa in humans [22]. In the TRAMP model, Simian Virus-40 T-antigen (SV-40 Tag) is under direct transcriptional control of the probasin promoter allowing androgen-regulated protein expression specific to the prostate epithelium. The TRAMP model created in 1996 by Greenberg et al. [22] has been used extensively over the past decade in PCa chemoprevention studies [6,23–27]. For this study, we hypothesized that dietary EGCG would protect against HG-PIN, the precursor to PCa, and spontaneously developing prostate adenocarcinoma by regulating sex steroid receptor, IGF-1 signaling, and COX-2 and iNOS in the prostate.

MATERIALS AND METHODS

Animals and Treatments

Animal care and use were conducted according to established guidelines approved by the National Institutes of Health (NIH) and the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (UAB). Breeders were purchased from the NCI Mouse Repository (Cancer Research Center, Frederick, MD) and used to develop our colony. Heterozygous transgenic (SV-40 Tag) females were crossed with non-transgenic C57BL/6 males to generate heterozygous transgenic (SV-40 Tag) male offspring for our studies (TRAMP C57BL/6 females X C57BL/6 male breeders). Mice were housed in rooms maintained at 24 ± 1C with a 12 hr light-dark cycle. At 3 weeks of age, offspring were tail-clipped and weaned. Mouse-tail DNA was extracted using a DNeasy Tissue Kit (Qiagen, Valencia, CA) and PCR was performed to determine the presence of the SV-40 Tag transgene [22,25].

Twelve weeks of age was selected to evaluate the influence of EGCG on early-stage PCa, specifically the transition from HG-PIN to well-differentiated carcinoma. Twelve weeks of age, a time point prior to heterogeneous tumor formation, is ideal for investigating the mechanisms of action in the TRAMP model. At

12 weeks of age, TRAMP mice display pre-malignant lesions but have not developed tumors that have the capacity to compromise oxygen and nutrients to the tissue. To investigate the effects of EGCG on apoptosis, cell proliferation, and protein biomarkers, animals were killed at 12 weeks of age and dorsolateral (DLP) and ventral prostates (VP) were excised, collected, weighed, flash-frozen in liquid nitrogen, and stored at −80C until time of analysis. Since it is unclear which lobe in the rodent resembles the human peripheral zone, the prostatic location in humans where PCa normally occurs [28], we chose to analyze both the DLP, which has historically been referred to as the homologue of the human prostate and the VP for mechanism of action studies. Based on our previous studies, 28 weeks of age was selected to evaluate the ability of EGCG to prevent late-stage poorly differentiated PCa. At that age mice were not set up for mechanistic studies since a large percentage of these animals develop macroscopic tumors in the prostate and the separation of lobes is not possible. Blood serum was collected at both 12 and 28 weeks of age.

Dietary treatment with EGCG was initiated at 5 weeks of age and continued until sacrifice at 12 or 28 weeks of age. Animals received powdered phytoestrogen-free AIN-76A diet (Harlan Teklad Global Diets, Wilmington, DE) and tap water (control) or 0.06% EGCG (treatment) ad libitum. The EGCG dose was extrapolated from the PCa chemoprevention study carried out by Gupta et al. that used 0.1% GTPs of which 62% of those green tea catechins were EGCG [6]. EGCG was prepared and administered fresh daily and the feeding regimen mimics an approximate human consumption of six cups of green tea [29]. Food and water consumption, as well as body weights were monitored at 6, 12, 18, 24, and 28 weeks of age. Tumor palpation was initiated at 18 weeks of age, initially once per week, and then twice per week starting at 24 weeks of age until sacrifice.

Chemicals

EGCG (Roche, Basel, Switzerland), extracted from non-fermented *Camellia sinensis* and tested as 93% pure by HPLC, was mixed at 0.06% in tap water. Amber-colored bottles were used to prevent oxidation caused by light.

Histopathology

At necropsy, all organs were examined for gross abnormalities in control and EGCG-treated animals. Metastasis to the bone, abdominal wall, lymph nodes, liver, kidney, and lung was investigated. Prostate, testes, seminal vesicles, and if present, tumor weights were also recorded. The entire urogenital tract containing the DLP, VP, and urethra were placed in cassettes, immersed in 10% formalin, dehydrated in a series of alcohol dilutions, fixed in xylene, embedded in paraffin wax, sliced into 5 μm sections, and placed on microscope slides as described by Folkvord et al. [30]. Sections were stained with hematoxylin and eosin prior to histopathological examination. Dr. Isam Eltoum, a Board Certified Pathologist, blindly scored each coded sample using the following grading scale developed specifically for rodents: Grade 1 (non-cancerous), Grade 2 (low-grade PIN), Grade 3 (HG-PIN), Grade 4 (well-differentiated lesion), Grade 5 (moderately differentiated lesion), or Grade 6 (poorly differentiated lesion) [24,25].

Cell Proliferation

Prostate tissue (DLP and VP) were dissected from 12-week-old TRAMP mice (6 controls, 6 EGCG-treated) and processed for detecting Ki-67, a marker of cell proliferation. Briefly, paraffin-embedded tissue sections (5 μm) on glass slides were deparaffinized in xylene and rehydrated in a gradient of alcohols. Samples were boiled in Antigen Unmasking Solution (Vector, Burlingame, CA) for 15 min and then cooled under tap water. H_2O_2 (3%) was used to inhibit endogenous peroxidase activity by incubation at room temperature for 10 min. Blocking was accomplished using 2.5% Normal Horse Serum from the ImmPRESS^{TM} Reagent Kit (Anti-Mouse Ig) (Vector). Next, monoclonal mouse anti-rat Ki-67 antigen, Clone MIB-5 antibody (DakoCytomation, Carpinteria, CA) diluted in PBS with 1% BSA was applied overnight to the specimens and followed by washes in PBS. ImmPRESS^{TM} Reagent secondary antibody was applied to the samples for 30 min followed by washes in PBS. Chromogen, diaminobenzidine (DAB) (Vector) was applied to samples for 10 min followed by a wash in tap H_2O for 5 min. To counterstain, hematoxylin QS (Vector) was applied to the specimens for 1 min, followed by a wash in tap H_2O. Specimens were immersed in a series of graded alcohols, placed in xylene, and mounted with coverslips using Mounting Media (Vector). The slides were viewed using a Nikon Labophot-2 microscope (Nikon Corporation, Tokyo, Japan) and digitally recorded using a Nikon 8.0 Mega Pixels CoolPix 8700 Digital Camera (Nikon). For Ki67 quantitation, epithelial cells were counted using Image J software (Image J, NIH). The DLP and VP were analyzed separately (a minimum of 2,000 cells counted per lobe per slide; six samples each). The epithelial cells staining positive (brown) for Ki67 were counted as well as the non-proliferative epithelial cells (stained blue). The proliferative index was defined as the number of positively stained cells divided by the total number of cells counted X 100. Twenty eight-week-old TRAMP

prostate tumor with and without Ki67 primary antibody was used as positive and negative controls, respectively.

Apoptosis

Apoptotic bodies were counted from tissue sectioned-slides stained with hematoxylin and eosin to assess apoptotic index in 12-week-old control or EGCG-treated TRAMP mice (6 each). The morphological criteria for identifying apoptosis were based on previously published studies [31–33]. Briefly, apoptotic bodies consist of rounded masses of cytoplasm containing hyperchromatic pyknotic nuclei, dense chromatin fragments, and/or cellular fragments. Apoptotic bodies are usually surrounded by a clear halo. Only structures with unequivocal features of apoptosis were counted. Each slide was evaluated using a Nikon Labophot-2 light microscope (Nikon), digitally recorded using a Nikon 8.0 Mega Pixels CoolPix 8700 Digital Camera (Nikon), and counted using Image J software acquired from the National Institute of Health (NIH). The DLP and VP were analyzed separately by counting at least 2,000 cells per prostate lobe. The epithelial cells displaying apoptotic bodies were counted as well as the non-apoptotic epithelial cells. An apoptotic index was defined as the number of apoptotic bodies divided by the total number of cells counted X 100.

SV-40 Tag

Immunohistochemistry (IHC) was employed to measure SV-40 Tag expression in the DLP and VP of 12-week-old control ($n = 6$) and EGCG-treated ($n = 6$) TRAMP mice. SV-40 Tag expression was semi-quantitated and localization was evaluated as described previously [34]. SV-40 Tag was measured to determine if the effects of EGCG was due to a direct biological effect on the prostate or an indirect effect caused by an alteration in transgene expression. Non-transgenic C57/BL6 mouse prostate and rat mammary gland were used as negative controls.

Immunoblot Analyses

The protein expression levels of biomarkers were measured by western blot analysis as described previously [35]. The following primary antibodies were purchased from commercial sources: AR (Santa Cruz Biotechnology, Santa Cruz, CA, SC-816), ER-α (Santa Cruz, SC-542), insulin-like growth factor-1 receptor (IGF-1R) (Santa Cruz, SC-712), insulin-like growth factor-binding protein 3 (IGF-BP3) (Santa Cruz, SC-9028), phospho-ERKs 1 and 2 (Cell Signaling Technology, Danvers, MA, #9101S), total-extracellular signal-regulated kinases 1 and 2 (total-ERKs 1 and 2) (Cell Signaling, #9102), cyclooxygenase-2 (COX-2) (Santa Cruz, SC-1745), and inducible nitric oxide synthase (iNOS) (Santa Cruz, SC-651). SuperSignal West Dura Chemiluminescence (Pierce, Rockford, IL) was applied to detect the proteins of interest. The relative intensity of the bands was measured using VersaDoc Imaging System (BioRad, Hercules, CA). The use of Kaleidoscope Precision Plus Protein and Pre-stained SDS–PAGE Broad Range standards (BioRad) as well as positive controls aided in correctly identifying the proteins of interest.

Enzyme Linked Immunosorbent Assay (ELISA)

We quantitated IGF-1 levels in the prostate by ELISA as described by Crowther et al. [36]. Prior to analysis, kinetic curves were set up to establish zero order kinetics. For each sample, 1 μg of protein was diluted in 100 μl of coating solution (10 mM PBS, pH 7.2) and applied to a 96-well Nunc-Immuno plate (Nage Nunc International, Rochester, NY). Next, overnight incubation at room temperature and a series of washes with $1\times$ PBS + 0.05% Tween-20 (BioRad) were implemented. To block extraneous binding sites, PBS + 1% BSA was applied for 1 hr and then washed. Rabbit polyclonal IGF-1 primary antibody (Santa Cruz, SC-9013) diluted in 10 mM PBS + 1% BSA was added and incubated for 2 hr. After washing with PBS, HRP-conjugated anti-rabbit secondary antibody diluted in PBS + 1% BSA was incubated for 4 hr at room temperature. Following a series of washes, the reaction was incubated with the ImmunoPure TMB Substrate Kit (Pierce) and stopped using 2N H_2SO_4. Samples were run in duplicate and the absorbance at 450 nm was read in an OPTI max Microplate reader (Molecular Devices, Sunnyvale, CA). Mouse liver with and without IGF-1 primary antibody was used as a positive and negative control, respectively.

Blood Serum Hormone Concentrations

Serum total testosterone (bound and unbound), dihydrotestosterone (DHT), and estradiol concentrations were measured in the blood serum of 12- and 28-week-old control and EGCG-treated transgenic animals using radio-immunoassays (Diagnostic Systems Laboratories, Webster, TX) as described by the manufacturer. All samples were run in duplicate with 8 samples per group by Dr. John Mahan (OB/GYN Department, UAB, Birmingham, AL).

Polyphenol Concentrations in Blood Serum

Whole blood was collected from 12-week-old mice at time of sacrifice and centrifuged at 2,300 rpm for

10 min to collect serum. EGCG was extracted from the serum and measured on a 4000 Q TRAP® LC/MS/MS System (Applied Biosystems, Foster City, CA). Serum extractions were carried out as described previously [37,38]. Apiginin, 4-methylumbelliferone, and phenolphthalein glucuronide were added as internal standards. For EGCG serum analysis, 1% acetic acid in 100% methanol was used after incubation with internal standards and β-glucuronidase enzyme. Samples were reconstituted in 80% methanol prior to LC/MS/MS injection. Control serum from animals exposed to AIN-76A diet and tap water only was used as negative controls. Values were reported in molarity (nM).

Statistics

Fisher's exact test was used to evaluate histopathological grade frequencies among treatment groups. Statistical comparisons were performed using two-sample Student *t*-test assuming unequal variances for western blot analysis and ELISA. $P < 0.05$ was considered to be significant.

RESULTS

EGCG in the Diet Suppresses Early Stage PCa in TRAMP Mice

At 12 weeks of age, dietary EGCG significantly reduced the incidence of HG-PIN (Grade 3) from 100% to 17% in the VP of TRAMP mice (Table I). The suppression of HG-PIN (Grade 3), the precursor to prostate adenocarcinoma, was accompanied by a delay in progression of low-grade PIN (Grade 2) from 0% in controls to 83% in EGCG-treated animals. In the DLP of 12-week-old transgenic mice, EGCG did not have a protective effect. At 28 weeks of age, 67% of the TRAMP mice had carcinomas (Grades 4–6), including 23% having poorly differentiated tumors (Grade 6), therefore making it difficult to evaluate the VP from the DLP (Table II). Therefore, the complete urogenital tract/tumor was evaluated as one entity. At this age (28 weeks), there was no significant effect on all pathological stages from EGCG treatment. There was no statistical change in latency, number of palpable tumors per animal, tumor weight, or number of liver, kidney, lung, or lymph node metastases between the control- and EGCG-treated animals.

Transgene Expression was not Altered by EGCG Treatment

At 12 weeks, SV-40 Tag protein expression was investigated to determine if the effect of EGCG on histopathology and biomarkers was due to regulation of the probasin promoter. There was no EGCG treatment effect on SV-40 Tag expression in the DLP and VP epithelia (Fig. 1A–D).

Dietary EGCG Inhibits Epithelial Cell Proliferation and Increases Apoptosis in the Prostates of TRAMP Mice

To investigate the mechanisms of action of EGCG in preventing HG-PIN, we chose 12 weeks as the age to investigate since this is the age at which 100% of the untreated transgenic mice developed HG-PIN. As demonstrated by the Ki67 assay, cell proliferation was similar in VP and DLP of control-treated mice, with proliferative indices of 13 and 14 respectively (Fig. 2A–E). EGCG significantly decreased epithelial cell proliferation by 54% in the VP. There was change in cell proliferation in the DLP from EGCG treatment (Fig. 2A–E). The apoptotic index was twice as high in the DLP as in the VP of control-treated animals (Fig. 2F). Analysis of apoptosis showed that controlled cell death was significantly increased in the VP (fivefold; 394%), but not in the DLP (Fig. 2F).

TABLE I. Histopathological Analysis of the DLP and VP of 12-Week-Old TRAMP Mice Fed AIN-76A Diet and Tap Water (Control) or 0.06% EGCG in Tap Water Starting at 5 Weeks of Age

			Grade level					
Treatment	*n*	Lobe	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)
Control	6	VP	0	0	100	0	0	0
EGCG	6	VP	0	83*	17*	0	0	0
Control	6	DLP	0	0	100	0	0	0
EGCG	6	DLP	0	0	100	0	0	0

Samples were given a score of (1) normal tissue, (2) low-grade PIN, (3) high-grade PIN, (4) well-differentiated tumor, (5) moderately differentiated tumor, and (6) poorly differentiated tumor depending on the presence and progression of lesions. Results are the percentage of mice as a function of the pathological score.
*$P < 0.05$ compared to control treatment.

TABLE II. Histopathological Analysis of the Urogenital Tract of 28-week-old TRAMP Mice Fed AIN-76A Diet and Tap Water (Control) or 0.06% EGCG in Tap Water Starting at 5 Weeks of Age

		Grade level					
Treatment	n	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)
Control	53	0	0	34	42	2	23
EGCG	25	0	0	32	44	4	20

Samples were given a score of (1) normal tissue, (2) low-grade PIN, (3) high-grade PIN, (4) well-differentiated tumor, (5) moderately differentiated tumor, and (6) poorly differentiated tumor depending on the presence and progression of lesions. Results are the percentage of mice as a function of the pathological score.

AR, but not ER-α is Differentially Regulated in EGCG-Treated TRAMP Mice

Since early stage PCa is usually considered androgen dependent, we measured AR by western blot analysis. In the VP, AR protein expression was significantly reduced by 51% (Fig. 3), but there was no difference in AR in the DLP from EGCG treatment. EGCG in the water did not alter ER-α in either the VP or DLP (Fig. 3). Measurement of sex steroid hormones in blood serum showed that testosterone, estradiol, and DHT concentrations were not significantly different between control- or EGCG-treated mice at 12- and 28-week of age (data not shown).

IGF-1 Signaling Proteins Are Differentially Regulated in the Prostates of TRAMP Mice Following EGCG Treatment

Dietary EGCG significantly reduced IGF-1 protein levels in the VP by 30% and in the DLP by 31% (Fig. 4). In contrast, IGF-1R protein expression was significantly down-regulated (42%) in the VP, but remained unchanged in the DLP (Fig. 4). The protein expression of IGF-BP3, the most abundant IGF-1 binding protein, was not significantly different in the VP or DLP (data not shown). In addition, we investigated the effect of EGCG on specific protein expressions in the liver, the major site of IGF-1 production. IGF-1, IGF-1R, and

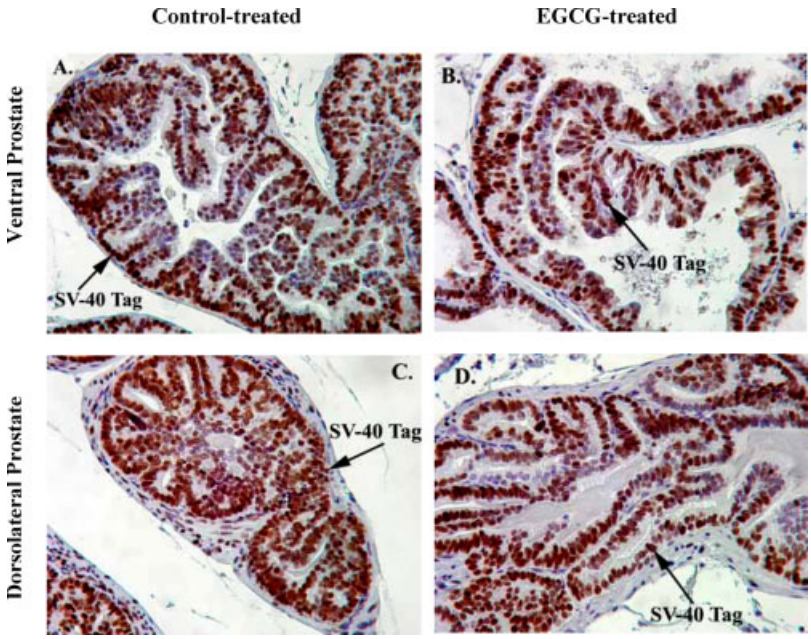


Fig. 1. Immunohistochemical staining of SV-40 Tag (40× magnification) in the VP (**A**,**B**) and DLP (**C**,**D**) of 12-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. Arrows exemplify (brown) positive prostatic epithelial cells expressing SV-40 Tag (transgene marker). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

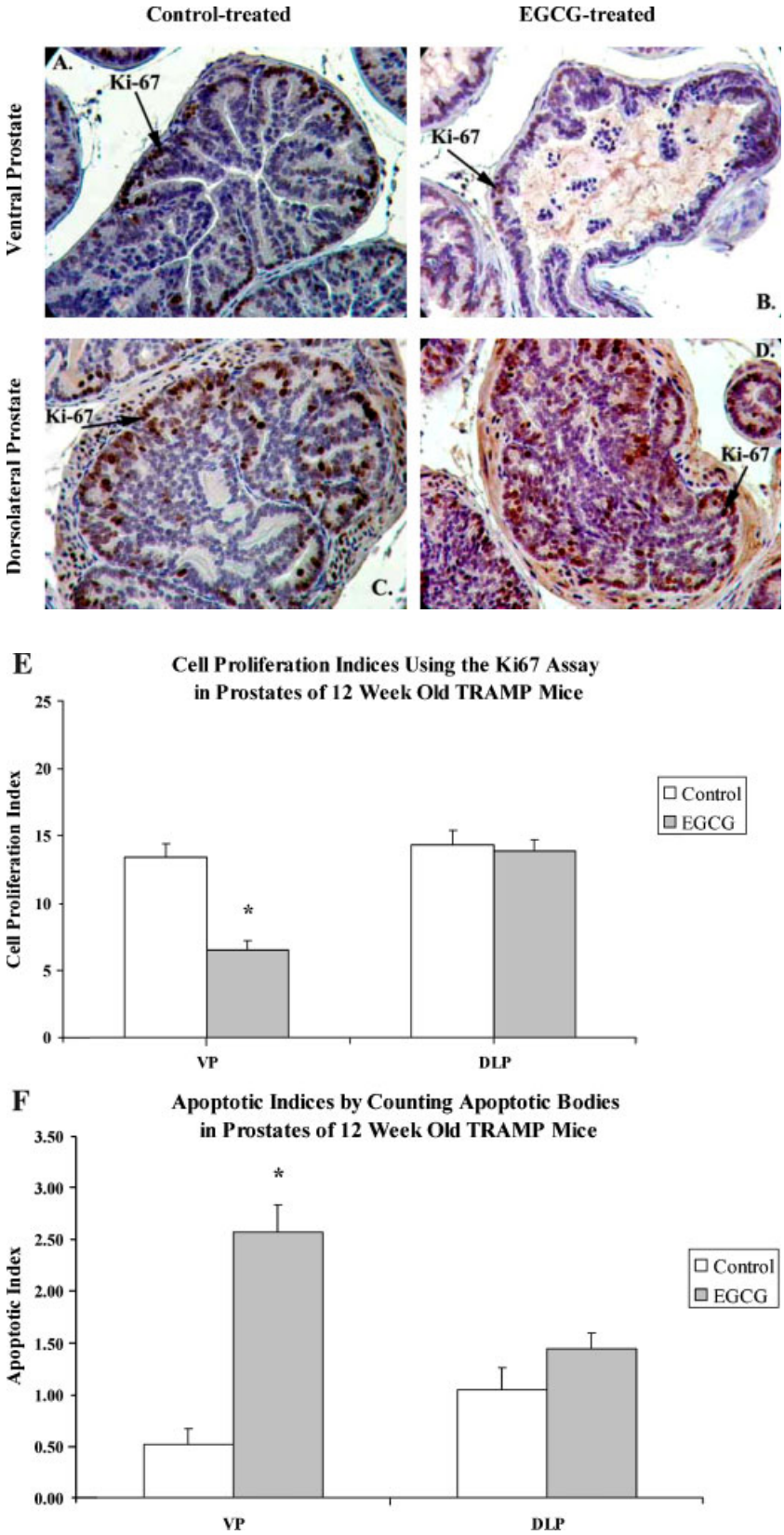


Fig. 2. Cell proliferation and apoptosis. Immunohistochemical staining of Ki-67 (40× magnification) in the VP (**A**,**B**) and DLP (**C**,**D**) of 12-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. Arrows show (brown) positive prostatic epithelial cells expressing Ki-67 (cell proliferation marker). **E**: Data represent the cell proliferative indices and (**F**) apoptotic indices. *$P < 0.05$ compared to control treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

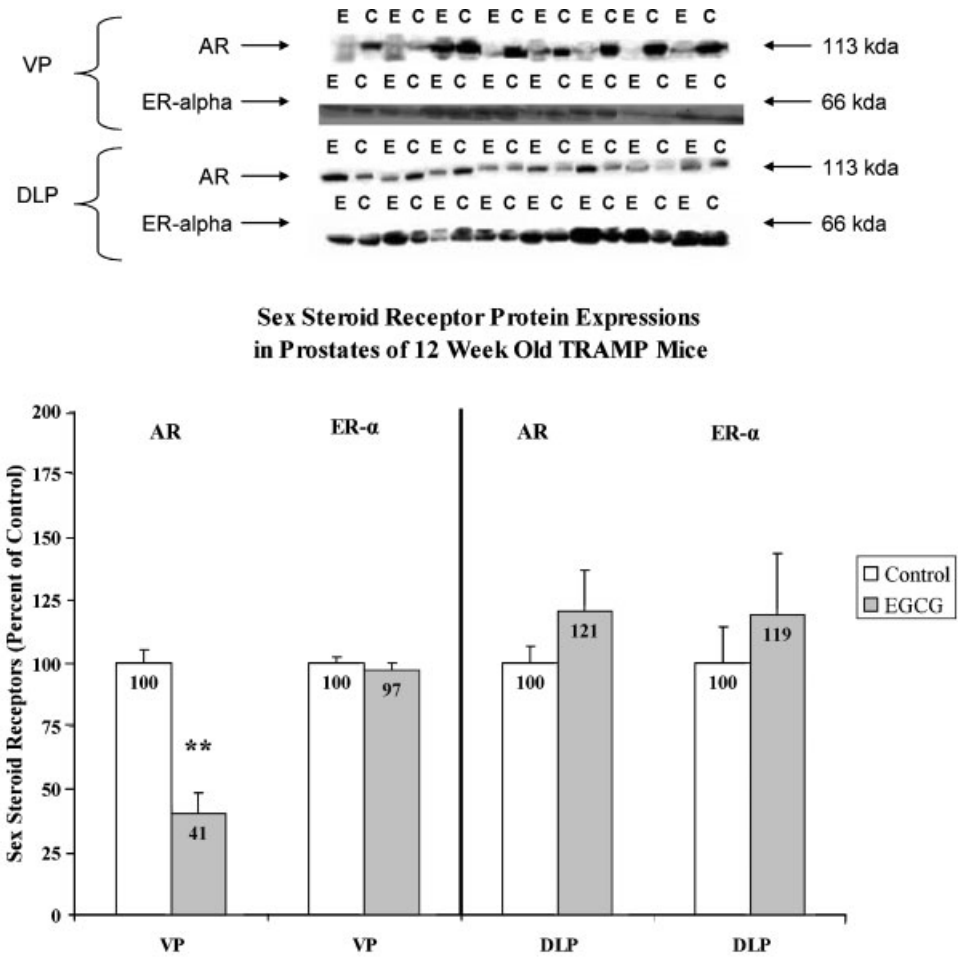


Fig. 3. AR and ER-α protein expression in VP and DLP of 12-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. Upper bands depict western blots for AR and ER-α and the lower figure is a graph of densitometry measurements from these western blot analyses. Each sample consisted of three pooled prostates and each group contained eight samples. Densitometry values for control mice were set at 100. $^{**}P < 0.001$ compared to control treatment.

IGF-BP3 protein expressions did not differ in the liver between control- and EGCG-treated mice (data not shown).

EGCG Down-Regulates Phospho-ERKs 1 and 2 Protein Expressions in the Ventral Prostates of TRAMP Mice

Protein tyrosine kinases, ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK) are downstream effectors of the IGF-1 signaling pathway and when activated can lead to cell proliferation. Phospho-ERKs 1 and 2 (activated forms) protein expressions in the VP were decreased by 45% and 57%, respectively (Fig. 5). On the other hand, EGCG did not alter phospho-ERKs 1 and 2 protein expressions in the DLP (Fig. 5). Total-ERKs 1 and 2 (phosphorylated and unphosphorylated) remained unchanged in DLP and VP (data not shown).

EGCG Reduced Both COX-2 and iNOS Protein Expression in the Ventral Prostate

We investigated COX-2 and iNOS, enzymes that mediate inflammatory processes and are associated with carcinogenesis, by western blot analysis. COX-2 and iNOS were found to be significantly decreased (24% and 77%, respectively) in the VP of EGCG-treated mice (Fig. 6). Again, following the same pattern as seen in sex steroid receptor and growth-factor signaling proteins, COX-2 and iNOS were not altered in the DLP of EGCG-treated animals when compared to control (Fig. 6).

EGCG in Tap Water is Well-Tolerated but Only Reaches Low Nanomolar Concentrations in the Blood Serum

EGCG at 0.06% in the drinking water did not show evidence of toxicity. There was no significant difference

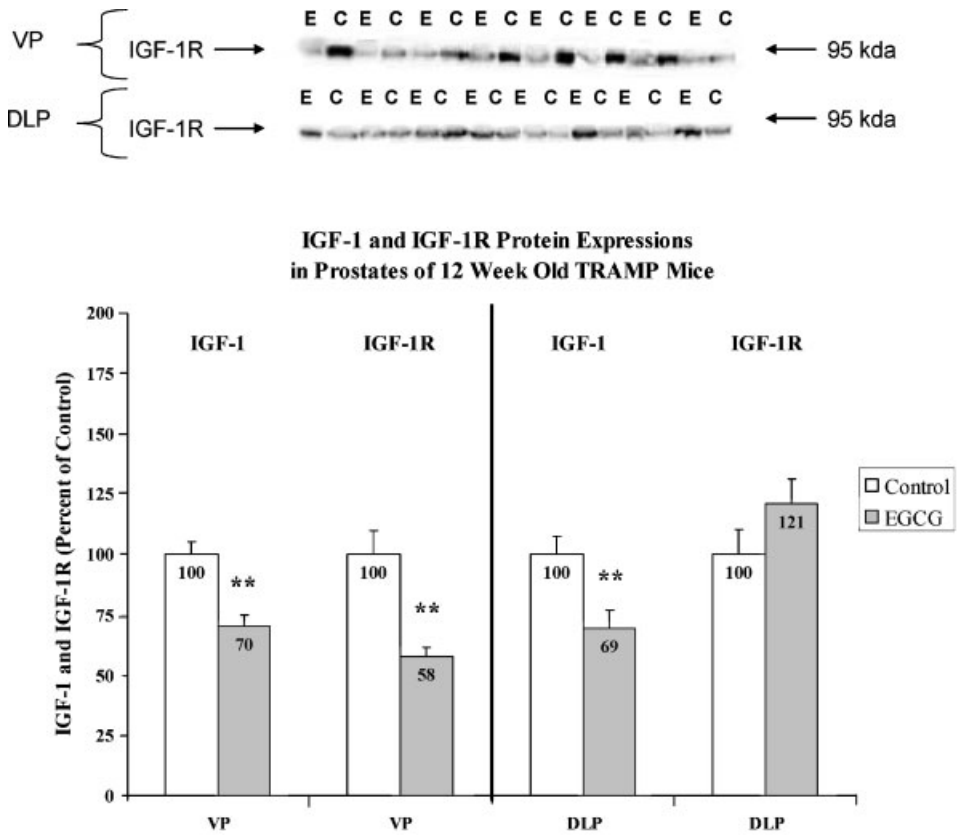


Fig. 4. IGF-1 and IGF-1R protein expressions in VP and DLP of 12-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. IGF-1 protein expression was determined via ELISA. Upper bands depict western blots for IGF-1R and the lower figure is a graph of densitometry measurements from these western blot analyses. Each sample consisted of three pooled prostates and each group contained eight samples. Densitometry values for control mice were set at 100. $^{**}P < 0.01$ compared to control treatment.

in food or water intake in EGCG-treated mice when compared to controls. Likewise, EGCG was well-tolerated and there was no significant change in the body weights of EGCG-treated mice compared to controls at 6, 12, 18, 24, and 28 weeks of age (data not shown). At 12 weeks of age, there was no significant change in testes, DLP, VP, or seminal vesicle weights (data not shown). Prostate to body weight and testes to body weight ratios did not differ between EGCG and control-treated mice (data not shown). EGCG (0.06%) in tap water had low bioavailability and subsequently reached a steady state concentration of 22 ± 4 nM in the blood serum (data not shown).

DISCUSSION

Our lab has previously demonstrated that genistein [25,39], the most abundant polyphenol in soy, and trans-resveratrol (Harper, unpublished work), an active phytochemical component in red wine, can suppress PCa development in the TRAMP model as single dietary agents. Previously, Gupta et al. reported that 0.1% GTP could reduce palpable prostate tumors in 32-week-old TRAMP mice [6]. Histological changes were noted between control- and GTP-treated animals, but tumors were not scored by a pathologist. Since EGCG comprised 62% of the before-mentioned preparation, we provided 0.06% EGCG in tap water to our TRAMP mice to determine if the effects of GTP could be solely attributed to EGCG. Using this concentration, we found that EGCG suppressed HG-PIN in the VP, but not in the DLP, hence demonstrating lobe specificity. In the TRAMP model, HG-PIN starts to develop by 10 weeks of age, followed by invasive PCa at 18 weeks of age. Therefore, HG-PIN offers promise as an intermediate endpoint in PCa chemoprevention studies. The suppression of HG-PIN, the precursor to PCa, suggests that EGCG alone can act to slow the progression of PCa. However, we found that the EGCG treatment did not suppress PCa development in 28-week-old TRAMP mice when the entire prostate was evaluated histopathologically.

Our report reveals several interesting points. First, EGCG alone may not be responsible for GTP suppressing late stage prostate cancer in the TRAMP model. It is possible that it is another component of GTP or the multiple polyphenols in the green tea extract that act additively or synergistically to have a chemopreventive

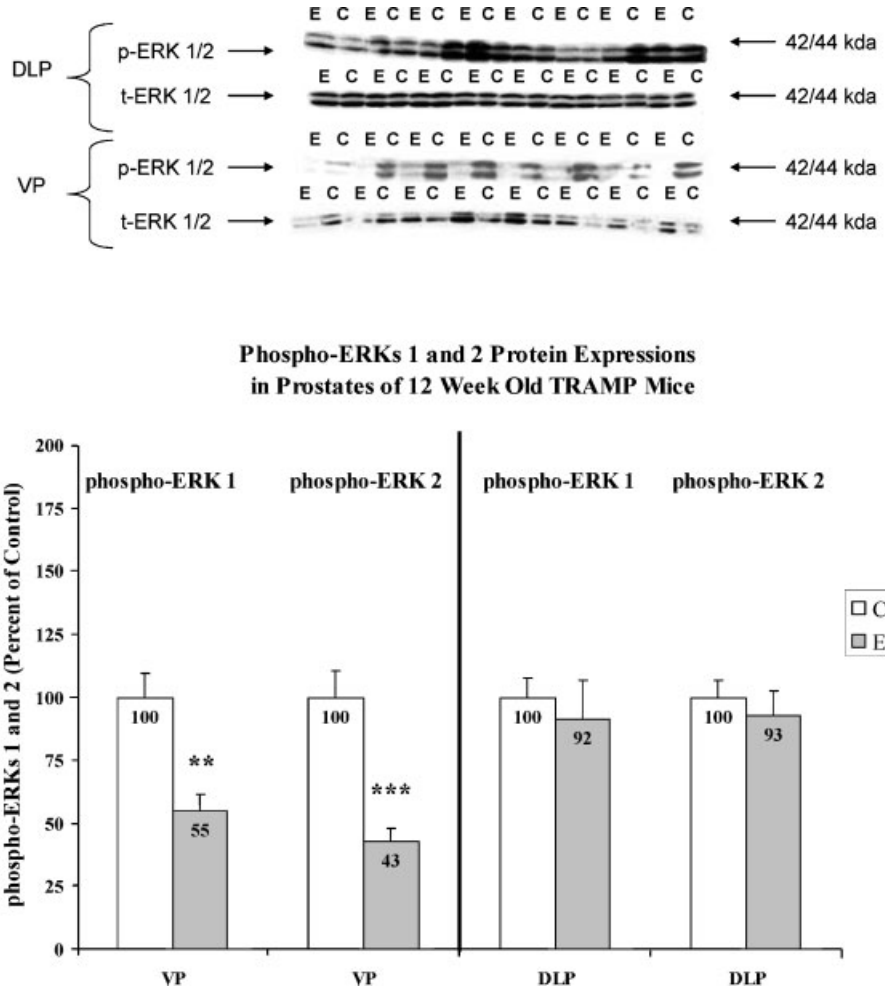
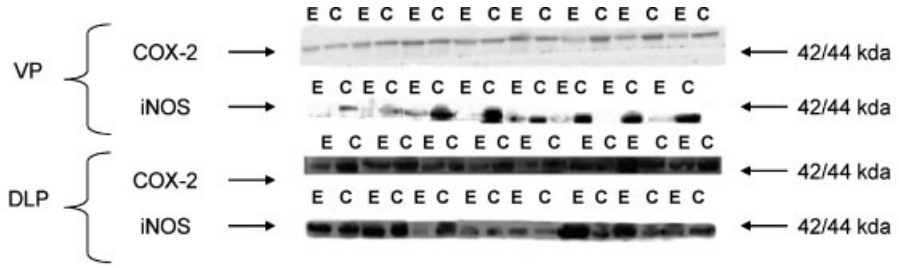


Fig. 5. Phospho-ERKs I and 2 protein expression in VP and DLP of I2-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. Upper bands depict western blots for Phospho-ERKS I and 2 and the lower figure is a graph of densitometry measurements from these western blot analyses. Each sample consisted of three pooled prostates and each group contained eight samples. Densitometry values for control mice were set at I00. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control treatment.

effect. Also, it is possible that the mixture of polyphenols in green tea may aid in stabilizing EGCG and slow its rapid metabolism. Other contributing factors to the differences seen between studies may include the use of different background strains [C57BL/6 and FVB] to generate the TRAMP colony and the low bioavailability of EGCG as a single agent. The nanomolar blood serum concentrations observed in our study is consistent with those concentrations found in other studies using 0.06% EGCG [40] and 0.1–0.6% GTPs [41]. Likewise, it has been shown that the pure EGCG has lower absorption and plasma levels and a higher excretion rate when given alone than when given in combination with other GTPs at the same concentration [42].

In reference to lobe specificity, that may explain why we do not see a suppression of prostate cancer at 28 weeks, that is, EGCG is effective only in suppressing prostate cancer development in the VP and not the DLP and by 28 weeks prostate cancer progression in the DLP has overwhelmed the VP. This is supported by the fact that we were no longer able to dissect the individual lobes at that age. EGCG molecular mechanisms of action support the latter.

Consistent with EGCG suppressing high grade PIN in TRAMP mice, EGCG decreased epithelial cell proliferation and increased apoptosis in the VP at 12 weeks of age. Investigation into molecular mechanisms, revealed that EGCG down-regulated AR by 51% in the VP, but not in the DLP. Down-regulated AR in the VP may provide a means of protection in the prostate. It is hypothesized that a reduction in AR decreases the potential for testosterone and DHT to signal through AR and cause prostate growth. In response, a plethora of PCa treatments have been designed to target AR via hormone therapy, including anti-androgens (i.e. bicaltumide, flutamide) and 5-α reductase inhibitors (i.e., Finasteride). Our findings support another study that



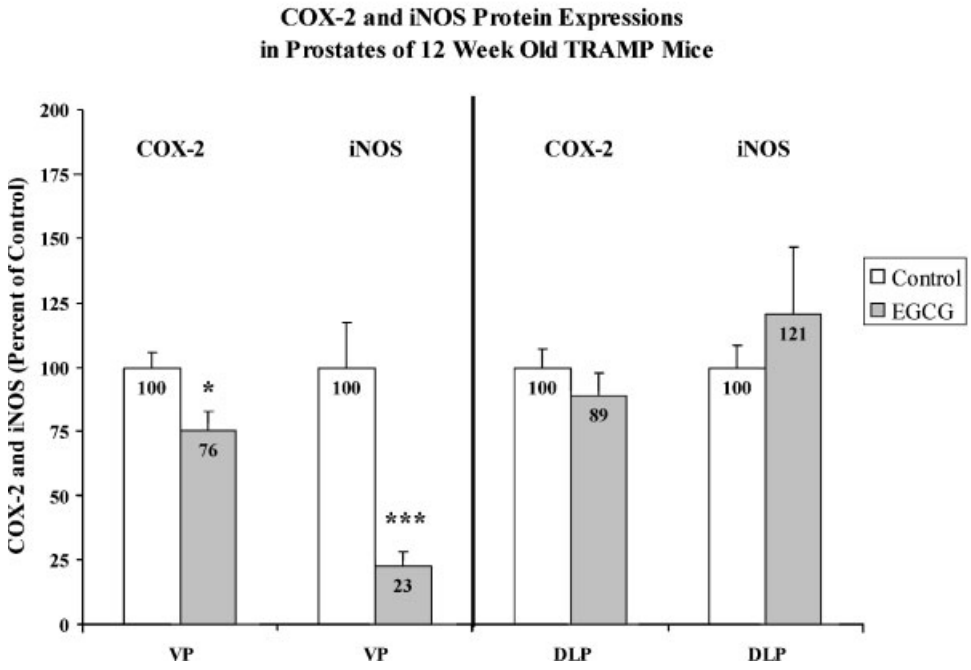


Fig. 6. COX-2 and iNOS protein expression in VP and DLP of 12-week-old TRAMP mice fed AIN-76A diet and tap water (Control) or 0.06% EGCG in tap water starting at 5 weeks of age. Upper bands depict western blots for COX-2 and iNOS and the lower figure is a graph of densitometry measurements from these western blot analyses. Each sample consisted of three pooled prostates and each group contained eight samples. Densitometry values for control mice were set at 100. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to control treatment.

showed the ability of EGCG to repress the transcription of the AR gene in LNCaP PCa cells [18]. To our knowledge, our study is the first to demonstrate AR regulation in the prostate using EGCG in an in vivo system. EGCG appears to regulate androgen action at the receptor level, since there was no alteration in testosterone and DHT in the blood. In addition, sex steroid receptor, ER-α, and its natural ligand, estradiol, was not modulated by EGCG and thus did not seem to play a significant role in its mechanism of action. It is well-known that estrogens play pivotal roles in normal growth, development, and differentiation of the prostate [43], but their role in the PCa etiology is not completely understood.

Not only was AR regulated in the prostate of TRAMP mice by dietary EGCG, but so was the IGF-1 signaling pathway. EGCG regulated the IGF-1 signaling pathway at the ligand and receptor level. Our results, demonstrated a decrease in IGF-1 in both prostate lobes with a concomitant decrease in the IGF-1R in the VP. Adhami et al. showed decrease in IGF-1 and an increase in IGF-BP3 in the DLP and blood serum of GTP-treated TRAMP mice [15]. Also, albeit in HT29 human colon cancer cells, a recent study described the role of EGCG as a tyrosine receptor inhibitor via its down-regulation of IGF-1R [44]. Although often debated, elevated blood levels of IGF-1 has been reported to be associated with an increased risk of PCa [45,46]. Therefore, EGCG may act to slow the progression of PCa by down-regulating IGF-1 signaling in the prostate. Studies of IGF-1R expression in early-stage prostate tumors revealed elevated levels of IGF-1R, while IGF-1R expression is reduced in advanced and metastatic PCa [47]. The down-regulation of IGF-1R expression in the VP of TRAMP mice at 12 weeks of age by EGCG also suggests a chemoprevention action.

To investigate the effects of EGCG downstream of AR and IGF-1 signaling, we measured phospho-ERKs

1 and 2, as well as proliferation and apoptosis. The sex steroid receptor and transcription factor, AR, and potent growth factor, IGF-1, can activate the MAPK pathway which subsequently can lead to the transcription of genes responsible for cell proliferation and apoptosis [48]. Cell proliferation and apoptosis are important endpoints that are often measured to determine if an agent is acting on the cell cycle to inhibit or enhance cell growth and death. In addition, agents that are able to attenuate MAPK, inhibit proliferation, and induce apoptosis are often considered attractive candidates for cancer prevention and therapy. In the current study, phospho-ERKs 1 and 2 were decreased in the VP of EGCG-treated mice. Our finding compliments a previous study that showed an inhibition of phospho-ERKs 1 and 2 in the DLP of GTP-treated TRAMP mice [15]. Phospho-ERKs 1 and 2 are up-regulated in transgenic mice with PCa when compared to their non-transgenic litermates [49]. Likewise, in our study, EGCG reduced cell proliferation by 54% and increased apoptosis by 394% in the VP of 12-week TRAMP mice. The entire prostate, with the DLP and VP measured as one entity, showed a similar but less drastic reduction. In support of these results, an array of in vitro studies has demonstrated a reduction in cell proliferation and an increase in apoptosis in both androgen-dependent and–independent PCa cell lines [50–58].

It is plausible to postulate that cross-talk between AR and the IGF-1 signaling pathway may have contributed to and potentiated the effects observed in downstream effectors, cell proliferation, and apoptosis. In LNCaP cells, an AR positive PCa cell line, androgens up-regulated IGF-1R expression and sensitized prostate cells to the biological effects of IGF-1 [59]. Other studies have provided evidence that IGF-1 and other growth factors can activate AR in the presence [60] or absence of androgens [17]. Therefore, a reduction in both AR and IGF-1 in the prostate may create a microenvironment with reduced potential for PCa growth and progression.

Because inflammation has been associated with PCa progression, we investigated if EGCG could regulate key proteins in this pathway. COX-2 and iNOS are two important enzymes that mediate inflammatory processes, but can also produce reactive oxygen radicals that can potentially damage DNA. COX-2 expression has been reported to be over-expressed in prostate adenocarcinoma [27], and excessive or aberrant iNOS expression has been implicated in pathogenesis of cancer [61]. Indeed, we showed a decrease in both COX-2 and iNOS expression in the VP of EGCG-treated animals. EGCG effectively reduced the potential for DNA damage and cancer progression in the prostate by inhibiting COX-2 and iNOS.

Our data illustrates a common theme in which EGCG as a single agent acts in a lobe-specific fashion in the rodent. EGCG reduced cell proliferation, induced apoptosis, and modulated PCa biomarkers in the VP only, with the exception of IGF-1 which was down-regulated in both the DLP and VP. Our data, with EGCG mediating most of its action via the VP, supports other studies that have shown EGCG to protect against PCa [34] and modulate inflammation and invasion of PCa [21] in the VP only in TRAMP mice. In the TRAMP model, the rapid and preferential development in the DLP [22] may mask the anti-tumor (anti-cancer) effects of EGCG on the VP at 28 weeks of age when evaluating the prostate as a whole. Future studies are aimed at evaluating the effects of EGCG at an intermediate age (i.e., 17 weeks) to clarify this.

CONCLUSIONS

Any reduction in the carcinogenesis process by a chemopreventive agent is a significant achievement and has the potential to prolong the lifespan and enhance the quality of life in men. EGCG as a single agent inhibited HG-PIN, the precursor to PCa, but not advanced, late-stage PCa in young adult TRAMP mice. EGCG was able to change biomarkers that are commonly associated with chemoprevention. Based on our data, we suggest that the decrease in AR, attenuation in the IGF-1 signaling pathway, reduction in COX-2 and iNOS, and down-regulation of downstream effectors, phospho-ERKs 1 and 2 may contribute to the reduction in cell proliferation and induction of apoptosis and hence provide a mechanism of action for EGCG suppressing PCa without toxicity.

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