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Oral Consumption of Green Tea Polyphenols Inhibits Insulin-Like Growth Factor-I–Induced Signaling in an Autochthonous Mouse Model of Prostate Cancer

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ABSTRACT

We earlier demonstrated that oral infusion of green tea polyphenols inhibits development and progression of prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Evidence indicates that elevated levels of IGF-I with concomitant lowering of IGF binding protein (IGFBP)-3 are associated with increased risk for prostate cancer development and progression. In this study, we examined the role of IGF/IGFBP-3 signaling and its downstream and other associated events during chemoprevention of prostate cancer by green tea polyphenols in TRAMP mice. Our data demonstrated an increase in the levels of IGF-I, phosphatidylinositol 3′-kinase, phosphorylated Akt (Thr-308), and extracellular signal-regulated kinase 1/2 with concomitant decrease in IGFBP-3 in dorso-lateral prostate of TRAMP mice during the course of cancer progression, *i.e.*, as a function of age. Continuous green tea polyphenol infusion for 24 weeks to these mice resulted in substantial reduction in the levels of IGF-I and significant increase in the levels of IGFBP-3 in the dorso-lateral prostate. This modulation of IGF/IGFBP-3 was found to be associated with an inhibition of protein expression of phosphatidylinositol 3′-kinase, phosphorylated forms of Akt (Thr-308) and extracellular signal-regulated kinase 1/2. Furthermore, green tea polyphenol infusion resulted in marked inhibition of markers of angiogenesis and metastasis most notably vascular endothelial growth factor, urokinase plasminogen activator, and matrix metalloproteinases 2 and 9. Based on our data, we suggest that IGF-I/IGFBP-3 signaling pathway is a prime pathway for green tea polyphenol-mediated inhibition of prostate cancer that limits the progression of cancer through inhibition of angiogenesis and metastasis.

INTRODUCTION

Prostate cancer ranks as the most common noncutaneous malignancy and the second leading cause of cancer-related deaths in American males (1). In the absence of satisfactory treatment options for prostate cancer, chemoprevention could be an effective approach to reduce the incidence the disease (2–8). In fact, prostate cancer represents an ideal candidate disease for chemoprevention because it is typically diagnosed in elderly men; therefore even a modest delay in the neoplastic development achieved through pharmacologic or nutritional intervention could result in substantial reduction in the incidence of the clinically detectable disease. Consistent with this notion, there is currently intense effort in identifying mechanism-based naturally occurring agents present in diet and beverages consumed by humans for their cancer chemopreventive and possibly cancer therapeutic efficacy against prostate cancer (2–8). Among these agents we (5–11 and references therein) and others (12–16 and references therein) have proposed polyphenols derived from green tea (GTP) as potential chemopreventive agents against prostate cancer, primarily because of their high intake by populations with reduced cancer incidence and their reported ability to inhibit proliferation and induction of apoptosis in prostate cancer cells in culture. We have earlier shown that oral infusion of green tea polyphenols to transgenic adenocarcinoma of the mouse prostate (TRAMP), a model in which cancer develops in a manner similar to human disease, at a human achievable dose (equivalent to 6 cups of green tea per day), inhibits the development of prostate cancer and its subsequent progression (8). An important observation from this study was the inhibition of serum IGF-I levels with concomitant increase in IGFBP-3 levels in green tea polyphenol-fed mice. This is consistent with observations in humans in which most case control studies have shown increased insulin-like growth factor (IGF)-I levels in men with prostate cancer compared with men with benign prostatic hyperplasia or controls (17–20). The increased levels of IGF could also contribute to the initiation and progression of prostate cancer because it is also known to induce vascular endothelial growth factor (VEGF), and it has been proposed that IGF is involved in the angiogenic switch leading to prostatic neovascularization (20). It is known that type I IGF receptor is a regulator of matrix metalloproteinase (MMP)-2 synthesis (21). Increased IGF signaling also enhances the expression of VEGF, urokinase plasminogen activator, and MMPs, which in turn closely correlate with tumor angiogenesis and metastasis (20–24). Understanding the molecular mechanisms of green tea polyphenol-mediated inhibition of prostate cancer is essential in devising rationale and mechanism-based chemopreventive approaches. Here, we show that green tea polyphenol-induced suppression of prostate cancer progression, metastasis, and angiogenesis may be mediated through inhibition of IGF and its downstream signaling pathway and suggest that IGF/IGFBP-3 pathway as a target for green tea polyphenol-mediated inhibition of prostate cancer progression in TRAMP.

MATERIALS AND METHODS

Animals. The male and female heterozygous C57BL/TGN TRAMP mice, Line PB Tag 8247NG, were purchased as breeding pairs from The Jackson Laboratory (Bar Harbor, ME). The animals were bred on same genetic background and maintained in the Animal Care Facility of Case Western University or University of Wisconsin, School of Medicine. Housing and care of the animals was in accordance with the guidelines established by the University's Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP × C57BL/6] F1 or as [TRAMP × C57BL/6]F2 offspring. Identity of transgenic mice was established by the PCR-based DNA screening as described previously (25).

Study Design and Green Tea Polyphenol Supplementation. Green tea polyphenols (green tea polyphenols >95% enriched preparation) were obtained from Natural Resources & Products (Charlottesville, VA). Chromatographic analysis of this mixture showed that it contains four major polyphenolic constituents: epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), and epicatechin (6%). Throughout the experiment, the animals were housed under standard animal housing conditions and had free access to laboratory chow *ad libitum*. As described previously (8), freshly prepared solution of 0.1% green tea polyphenols in tap water was supplied every Monday, Wednesday, and Friday to experimental animals as the sole source of drinking fluid for 24 weeks (green tea polyphenol-fed group), and the control (water-fed group) animals were supplied with the same tap water throughout the experiment. This feeding regimen is well tolerated by

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animals and has been used in mice in many previous studies from this and other laboratories and is equivalent to an approximate consumption of 6 cups of green tea per day by an average adult human (8). For each experiment, 20 male 8-week-old TRAMP mice were divided into two equal groups consisting of 10 animals in each group. Such an experiment with 20 additional mice was repeated. At each time point and at the termination of the experiment, the mice were killed by cervical dislocation, and the dorso-lateral prostate was removed under a dissecting microscope for biochemical and histologic analysis.

Preparation and Analysis of Tissue. The dorso-lateral prostate was excised and weighed, and a small portion was fixed overnight in 10% zinc-buffered formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin-embedded tissues and mounted on slides. As described previously (8), histologic sections were reviewed by light microscopy for the presence of prostate cancer and classified as well-differentiated (multiple epithelial mitotic figures and apoptotic bodies, invasive glands with stromal hypercellularity), moderately differentiated (many acini completely filled with tumor yet still forming some glandular structures), or poorly differentiated (sheets of malignant cells with little or no glandular formation) prostate cancer or atrophic glands only (no identifiable tumor deposits).

Immunoblot Analysis. The dorso-lateral prostate removed from both treated and control groups was homogenized in lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, and 10 mg/mL leupeptin (pH 7.4)] at 4°C to prepare cell lysates. The protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). Appropriate amount of protein (25–50 μg) was resolved over 8 to 14% Tris-glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibody of IGF-I, IGFBP-3 (Cell Signaling Technology, Beverly, MA), VEGF, urokinase-type plasminogen activator (uPA; Santa Cruz Biotechnology, Santa Cruz, CA), phosphatidylinositol 3′-kinase (PI3k), Akt, extracellular signal-regulated kinase 1/2 (ERK1/2; Transduction Laboratories, Lexington, KY), β-actin (Sigma, St. Louis, MO), MMP-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP) 1, and TIMP-2 (Chemicon International, Temecula, CA) in blocking buffer overnight at 4°C. The membrane was then incubated with appropriate secondary antibody horseradish peroxidase (HRP) conjugate (Amersham Biosciences Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit (Amersham Biosciences). Equal loading of protein was confirmed by stripping the membrane and reprobing it with monoclonal β-actin primary antibody (Santa Cruz Biotechnology) and appropriate secondary HRP conjugate.

Densitometric Analysis. Immunoblots were scanned by HP Precisionscan Pro 3.13 (Hewlett-Packard Co., Palo Alto, CA). Densitometry measurements of the scanned bands were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT). Data were normalized to β-actin or suitable loading controls and expressed as mean $\pm$ *SEM* followed by appropriate statistical analysis.

Immunohistochemical Analysis. Sections (4 μm) were cut from paraffin-embedded prostate tissues. Immunostaining was performed using specific antibodies with appropriate dilutions and was replaced with either normal host serum or block for negative controls, followed by staining with appropriate HRP-conjugated secondary antibodies. The slides were developed in diaminobenzidine and counter stained with a weak solution of hematoxylin stain as described previously (26). The stained slides were dehydrated and mounted in permount and visualized on a Zeiss-Axiophot DM HT microscope (Zeiss-Axiophot, Jena, Germany). Images were captured with an attached camera linked to a computer.

Gelatin Zymography. Gelatinolytic activity of uPA was assessed by performing gelatin zymography following a modified protocol described previously (27). Fifty μg of the prostate tissue lysate were dissolved in SDS sample buffer and run under nondenaturing conditions in a 12% SDS-PAGE in gels that also contained 1% copolymerized gelatin (Invitrogen, Carlsbad, CA). After electrophoresis, the gels were washed twice for 15 minutes in buffer containing 5 mmol/L calcium chloride, 1 μmol/L zinc chloride, 50 mmol/L Tris-HCl (pH 7.6), and 2.5% Triton X-100. The gels were then incubated overnight in the same buffer that also contained 0.2 mol/L NaCl, 0.02% sodium azide, and 10 mmol/L calcium dichloride. The enzymatic activity was visualized by staining the gels with a solution containing 50% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue and followed by destaining in 10% methanol and 10% acetic acid.

Enzyme-Linked Immunosorbant Assay for Vascular Endothelial Growth Factor. A sandwich enzyme immunoassay technique (mouse VEGF immunoassay, R&D Systems, Minneapolis, MN) was used to quantitate VEGF levels in the serum of TRAMP mice by following the manufacturer's protocol. In brief, standards, controls, and samples were pipetted into microtiter plate wells coated with an affinity-purified polyclonal antibody specific for mouse VEGF and incubated for 2 hours at room temperature. After incubation, wells were washed with a wash buffer to remove any unbound substances. This was followed by incubation in enzyme-conjugated polyclonal antimouse VEGF for 1 hour, substrate solution, and finally coloring reagent. Absorbance was read at 450 nm, and results were represented as VEGF levels in picograms per milliliter of serum.

Statistical Analysis. Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. To assess change in protein expression during the course of cancer progression, comparisons were made with water-fed animals of the preceding age. To access the effect of oral feeding of green tea polyphenols, comparisons were made with age-matched water-fed TRAMP mice. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Expression of Insulin-Like Growth Factor-I/Insulin-Like Growth Factor Binding Protein-3 during Progressive Stages of Prostate Cancer Development in Transgenic Adenocarcinoma of the Mouse Prostate Mice. Our earlier studies have shown an increase in serum IGF-I levels with concomitant decrease in serum IGFBP-3 levels in TRAMP mice during prostate cancer progression (8). Because IGFs are known to be produced locally by most tissues in which they act in an autocrine or a paracrine manner (28, 29), we further determined IGF-I and IGFBP-3 expression in the dorso-lateral prostate tissue of TRAMP mice of increasing age and cancer progression. A progressive increase in the IGF-I protein expression was observed in TRAMP mice as cancer progressed from not detectable cancer at 8 weeks to well-differentiated carcinoma at 16 weeks to moderately differentiated carcinoma at 24 weeks and finally to poorly differentiated carcinoma at 32 weeks (Fig. 1*A*). This increase in IGF-I expression was associated with concomitant decrease in the protein expression of IGFBP-3, the major binding protein for IGF-I, most notably in animals with moderately differentiated adenocarcinoma (24 weeks) and poorly differentiated adenocarcinoma (32 weeks; Fig. 1*A*). Densitometric analysis revealed that the progression of cancer in TRAMP mice was associated with a shift in IGF-I to IGFBP-3 ratio favoring cancer progression (Fig. 1*A*). Similar results were observed by immunohistochemical analysis of the prostate tissue of TRAMP mice (Fig. 1*A*). Mice with moderately differentiated (24 weeks) and poorly differentiated adenocarcinoma (32 weeks) exhibited strong staining for IGF-I, and this expression was more intense in mice with poorly differentiated adenocarcinoma. This staining was especially seen in the epithelia of prostatic acini and also in the stroma. In contrast, a significant lowering of IGFBP-3 expression was observed in mice with moderately and poorly differentiated adenocarcinoma (Fig. 1*A*).

Expression of Phosphatidylinositol 3′-Kinase/Akt and Extracellular Signal-Regulated Kinase during Progressive Stages of Prostate Cancer Development in Transgenic Adenocarcinoma of the Mouse Prostate Mice. Intrinsic induction of IGF-I can trigger multiple signal transduction pathways that include the mitogen-activated protein kinase (MAPK; ERK) pathway and the PI3k-dependent pathway implicated in the cell survival signals (29, 30). We, therefore, analyzed the expression of PI3k, Akt, and ERK1/2 in the dorso-lateral prostate of TRAMP mice. Similar to an increase in the expression of IGF-I, significant increase in the activated form of the p85 subunit of

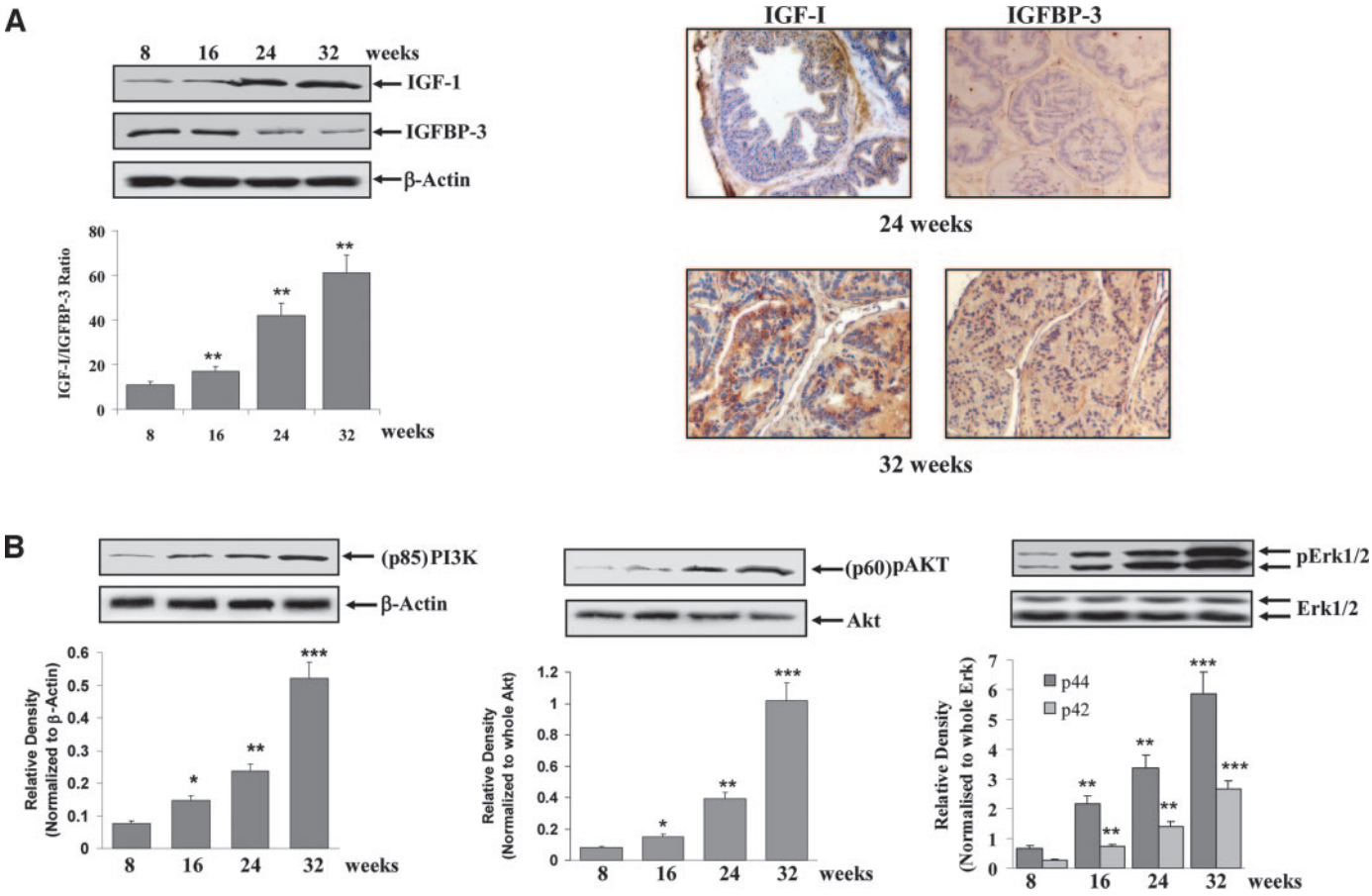

Fig. 1. Expression of IGF-I, IGFBP-3, PI3k, Akt, and ERK1/2 in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, protein levels of IGF-I and IGFBP-3 by immunoblotting and immunohistochemical analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of 8-, 16-, 24-, and 32-week-old TRAMP mice. Equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. Histogram indicates the ratio between IGF-I and IGFBP-3 obtained by densitometric analysis of the bands shown in *A* normalized to β-actin. Western blot analysis was conducted in five animals in each group, and only representative blots are shown. Immunostaining data were confirmed in two slides from five animals. Photomicrographs (magnification, ×40) represent immunohistochemical staining for IGF-I and IGFBP-3 in TRAMP with moderately differentiated (*24 weeks*) and poorly differentiated (*32 weeks*) adenocarcinoma. *B*, protein levels of PI3k, Akt, and ERK1/2 by immunoblot analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of 8-, 16-, 24-, and 32-week-old TRAMP mice. Blots were stripped and reprobed for analysis of β-actin, total Akt, and total Erk to confirm no change in the protein and also to serve for equal loading of the protein. Western blot analysis was conducted in five animals in each group, and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units ± *SEM*. **, $P < 0.01$; ***, $P < 0.001$ compared with water-fed TRAMP mice of the preceding age.

PI3k was observed (Fig. 1*B*). Furthermore, downstream of PI3k, we observed increased phosphorylation of Akt at Thr-308 and ERK1/2 (Fig. 1*B*) without any change in the expression of total Akt and ERK expression in the dorso-lateral prostate of TRAMP mice (Fig. 1*B*). When the data were analyzed densitometrically, we observed that the increase in the expression of these proteins was significantly higher ($P < 0.05$) compared with animals of the preceding age (Fig. 1*B*).

Effect of Green Tea Polyphenol Infusion to Transgenic Adenocarcinoma of the Mouse Prostate Mice on Insulin-Like Growth Factor-I Signaling Pathway. We have previously observed that green tea polyphenol infusion to TRAMP mice significantly lowered serum IGF-I and restored serum IGFBP-3 levels (8). As shown in Fig. 2*A* continuous green tea polyphenol infusion to TRAMP mice resulted in significant inhibition in the protein expression of IGF-I with concomitant restoration of IGFBP-3 levels in the dorso-lateral prostate tissue of TRAMP. Densitometric analysis of the bands suggested that IGF-I to IGFBP-3 ratios were inhibited by 70 to 83% ($P < 0.01$) by oral feeding of green tea polyphenols (Fig. 2*A*). These results were further confirmed by immunohistochemical analysis of IGF-I levels (Fig. 2*A*), indicating a significant decrease in IGF-I expression in green tea polyphenol-fed TRAMP mice. PI3k levels were significantly lowered by oral infusion of green tea polyphenols (by 67–79%, $P < 0.01$; Fig. 2*B*), and phosphorylation of Akt at Thr-308 was inhibited in green tea polyphenol-fed TRAMP mice (up to 65%, $P < 0.01$; Fig. 3*D*). We also observed significant inhibition (50–62%, $P < 0.01$) in the phosphorylation of ERK1/2 (Fig. 2*B*) in TRAMP mice that received green tea polyphenols as the sole source of drinking water.

Effect of Green Tea Polyphenol Infusion to Transgenic Adenocarcinoma of the Mouse Prostate Mice on Markers of Angiogenesis and Metastasis. One possible consequence of increased IGF-I levels is the promotion of angiogenesis because it is known that IGF-I can induce VEGF (31). We, therefore, determined the effect of green tea polyphenol infusion to TRAMP mice on VEGF expression. We observed decreased protein expression of VEGF in the dorso-lateral prostate of green tea polyphenol-fed TRAMP mice compared with the control water-fed group (Fig. 3*A*). This decrease was 34% ($P < 0.05$) at 16 weeks, 42% ($P < 0.01$) at 24 weeks, and 74% ($P < 0.001$) at 32 weeks of age as analyzed by densitometry (Fig. 3*A*). Although VEGF levels in the serum were higher in mice with moderately differentiated (24 weeks of age) and poorly differentiated (32 weeks of age) adenocarcinoma, its levels were found to be significantly lower in green tea polyphenol-fed TRAMP mice (by 43% at 24 weeks, $P < 0.01$, and 71% at 32 weeks, $P < 0.001$; Fig. 3*B*). We next determined the effect of green tea polyphenol infusion on the uPA expression, which is involved in the metastatic phenotype of many types of cancers (32).

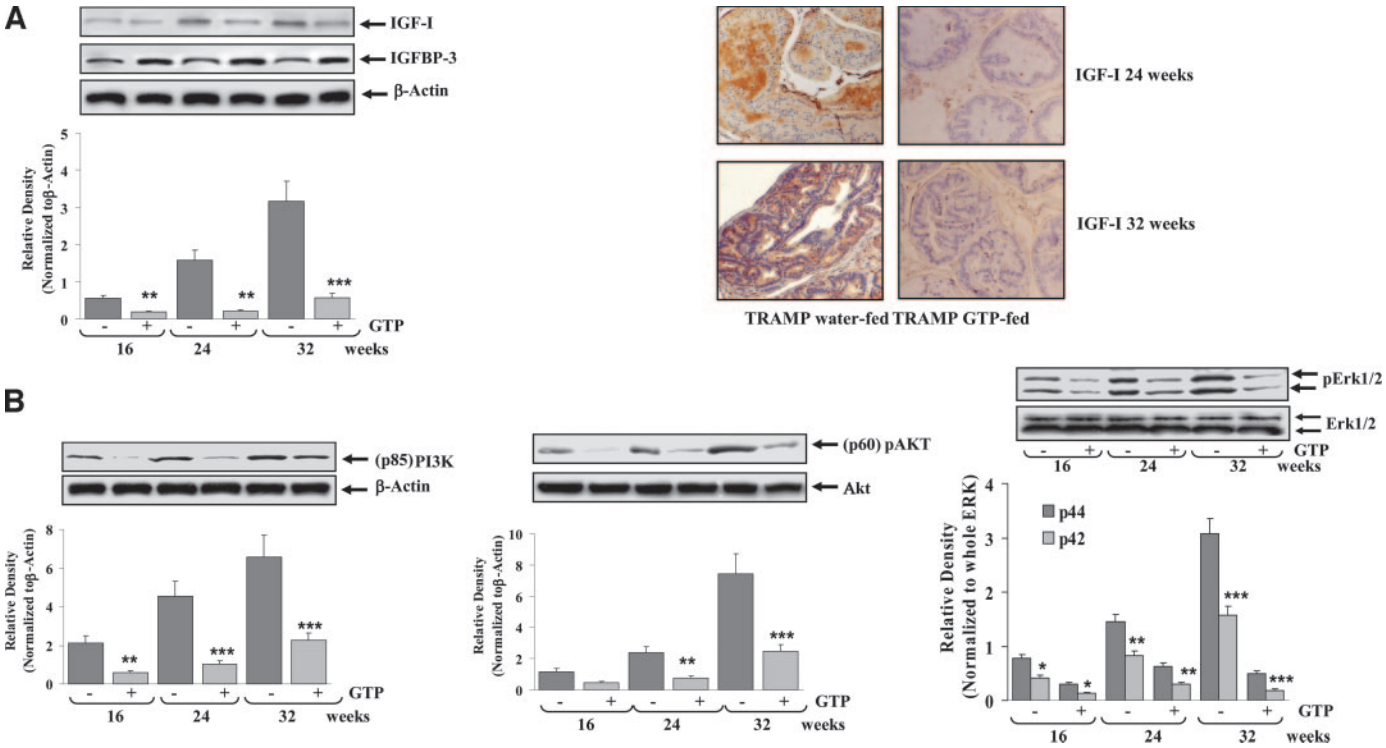

Fig. 2. Effect of green tea polyphenol infusion on IGF-I, IGFBP-3, and the PI3k, Akt, and ERK expressions in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, protein levels of IGF-I and IGFBP-3 by immunoblotting and immunohistochemical analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control TRAMP mice at 8, 16, 24, and 32 weeks of age and green tea polyphenol-fed TRAMP mice at 16, 24 and 32 weeks of age. In immunoblot analysis, the blots were stripped and reprobed for analysis of β-actin for equal loading of the protein. Western blot analysis was conducted in five animals in each group, and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units $\pm$ *SEM*. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with respective age-matched water-fed TRAMP mice. Representative photomicrographs (magnification, $\times$40) of immunohistochemical staining for IGF-I in water-fed control and green tea polyphenol-fed TRAMP mice with moderately differentiated (*24 weeks*) and poorly differentiated (*32 weeks*) adenocarcinoma. Immunostaining data were confirmed in two slides from five animals. *B*, protein levels of PI3k, phosho-AKT, and pErk1/2 by immunoblot analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control TRAMP mice at 8, 16, 24, and 32 weeks of age and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Blots were stripped and reprobed for analysis of β-actin, total Akt, and total Erk to confirm no change in the protein and also to serve for equal loading of the protein. Western blot analysis was conducted in five animals in each group, and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units $\pm$ *SEM*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with respective age-matched water-fed TRAMP mice.

As shown in Fig. 3*C* protein expression of uPA was significantly higher in green tea polyphenol-fed TRAMP mice with moderately differentiated (24 weeks of age) and poorly differentiated (32 weeks of age) adenocarcinoma. Continuous green tea polyphenol infusion to TRAMP mice resulted in significant inhibition in the expression of uPA. This inhibition was 31% ($P < 0.05$) in mice with moderately differentiated adenocarcinoma and 52% ($P < 0.01$) in mice with poorly differentiated adenocarcinoma (Fig. 3*C*). Interestingly, gelatin zymography for activity analysis revealed that the activity of uPA was inhibited by green tea polyphenols at all time points examined (Fig. 3*D*). Although increased uPA activity was observed in mice with moderately (24 weeks) and poorly (32 weeks) differentiated adenocarcinoma, a significant inhibition (47% to 64%, $P < 0.05$) in uPA activity was observed at all time points studied (Fig. 3*D*).

There is considerable evidence that one class of metalloenzyme, the matrix metalloproteinase (MMP), plays a key role in matrix degradation, thus allowing tumor dissemination (33 and references therein). Prostate cancers with invasive potential are known to secrete active forms of MMP-2 and MMP-9 in a process that is inhibited by TIMP-1 and TIMP-2 (34). In the next series of experiments, we investigated the tissue expression of MMPs and the TIMPs in control and green tea polyphenol-fed TRAMP mice. We observed increased protein expression of MMP-2 and MMP-9 (Fig. 4*A*) particularly in mice with moderately and poorly differentiated adeocarcinoma, time points that coincide with the initiation of metastasis. This increased expression was significantly inhibited (by 53 to 68%, $P < 0.01$) by continuous green tea polyphenol infusion to TRAMP mice. Densitometric analysis of the immunoblots revealed inhibition of MMP-2 (68% at 24 weeks, $P < 0.001$, and 53% at 32 weeks, $P < 0.01$) and MMP-9 (60% at 24 weeks, $P < 0.001$, and 65% at 32 weeks, $P < 0.001$) expression in TRAMP mice receiving 0.1% green tea polyphenols in drinking water (Fig. 4*A*). Similar results were observed by immunohistochemical analysis in which significant decrease in the protein expression of MMP-2 and MMP-9 (Fig. 4*B*) was observed in mice with poorly differentiated adenocarcinoma (32 weeks) after green tea polyphenol feeding compared with the control water-fed group of animals.

Endogenous levels of tissue inhibitors of metalloproteinases (TIMP) specifically inhibit the matrix metalloproteinases (34). The expression of MMPs and TIMPs has been proposed to be coregulated, and an imbalance between them has been shown to be an essential factor in the invasive phenotype of cancers (34). An increase in the TIMP expression was observed in TRAMP mice similar to that of MMP expression. Continuous green tea polyphenol infusion to TRAMP mice resulted in inhibition in the levels of TIMP-1 and TIMP-2 in mice with moderately (24 weeks, $P < 0.01$) and poorly (32 weeks, $P < 0.01$) differentiated adenocarcinoma (Fig. 5*A* and *B*). These observations were further analyzed and confirmed by immunohistochemistry of TIMP-1 and TIMP-2 in the dorso-lateral prostate tissues of water-fed TRAMP and green tea polyphenol-fed TRAMP animals with poorly differentiated adenocarcinoma. Interestingly, analysis of MMP to TIMP ratio in TRAMP mice indicates that the balance was shifted in such a way that favored MMP expression,

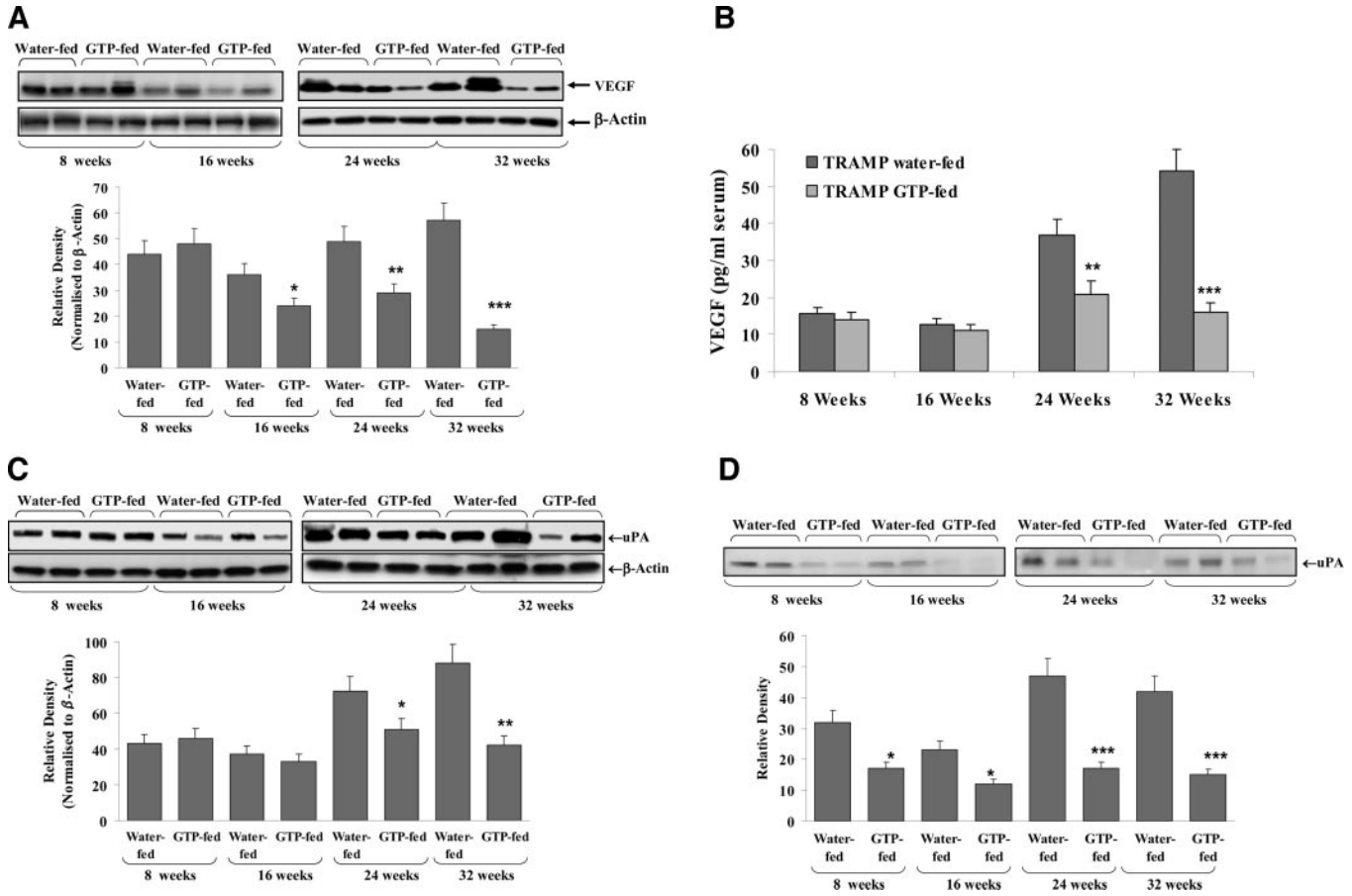

Fig. 3. Effect of green tea polyphenol infusion on VEGF and uPA levels in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, protein levels of VEGF by immunoblot analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control TRAMP mice at 8, 16, 24, and 32 weeks of age and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Histogram represents relative density data of the immunoblots in relative units ± *SEM*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with respective age-matched water-fed TRAMP mice. Western blot analysis was conducted in five animals in each group, and only representative blots for two animals are shown. *B*, enzyme-linked immunosorbant assay for quantitative evaluation of VEGF levels. As detailed in Materials and Methods, enzyme-linked immunosorbant assay was performed for quantitative evaluation of VEGF levels in the serum of water-fed control and green tea polyphenol-fed TRAMP mice at 8, 16, 24, and 32 weeks of age. For VEGF analysis, five samples of each group in triplicate were analyzed. Values represent mean ± *SEM*; **, $P < 0.01$; ***, $P < 0.001$ compared with respective water-fed control groups. *C*, protein levels of uPA by immunoblot analysis. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control TRAMP mice at 8, 16, 24, and 32 weeks of age and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Histogram represents relative density data of the immunoblots in relative units ± *SEM* of the bands. Western blot analysis was conducted in five animals in each group, and only representative blots for two animals are shown. *, $P < 0.05$; **, $P < 0.01$ compared with water-fed control TRAMP mice. *D*, gelatinolytic activity of uPA by gelatin zymography. As detailed in Materials and Methods, the uPA levels were determined in the dorso-lateral prostate of water-fed control TRAMP mice at 8, 16, 24, and 32 weeks of age and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Histogram represents relative density data of the zymogram in relative units ± *SEM*. Values represent mean ± *SEM*. *, $P < 0.05$; ***, $P < 0.001$ compared with respective water-fed control group.

whereas continuous green tea polyphenol infusion to TRAMP mice favored this ratio toward TIMP expression suggesting inhibition ($P < 0.01$) of MMP expression (Fig. 6*A* and *B*).

DISCUSSION

The limited available options for the treatment of prostate cancer have prompted the need for developing alternative strategies for the management of prostate cancer. Chemoprevention by the use of dietary or nontoxic synthetic agents has offered a viable option to block neoplastic inception or delay disease progression. Because prostate cancer is typically diagnosed in men ages 50 years and older, even a slight delay in the onset and subsequent progression of this disease through the use of dietary agents could have important health benefits. We have previously observed that oral infusion of a human achievable dose of green tea results in significant inhibition in the development and progression of prostate cancer along with increased survival in TRAMP (8). In this study, we examined the underlying mechanisms to understand whether IGF-I–induced signaling pathways are modulated by oral infusion of green tea polyphenols and further determined whether this feeding regimen inhibits the expression of molecules involved in metastasis and angiogenesis.

To begin with, we first evaluated levels of IGF-I and its binding protein IGFBP-3 in the dorso-lateral prostate of TRAMP mice at ages that were pathologically distinct from each other. We observed that as cancer progressed from undetectable cancer at 8 weeks to poorly differentiated adenocarcinoma at 32 weeks, the levels of IGF-I increased. This increase in IGF-I was associated with a concomitant decrease in its binding protein IGFBP-3, and relative assessment of the ratios of IGF-I and IGFBP-3 suggested a progressive and significant shift that favored increasing IGF-I levels. These results are consistent with previous observations in which prostate-specific IGF-I was found to be increased during prostate cancer progression in TRAMP mice (20). We have also previously observed increased elevated IGF-I levels with concomitant decrease in IGFBP-3 levels in the serum of TRAMP mice, and it has been suggested that the increase in serum IGF-I is probably due to prostatic response rather than due to a systemic response (20). It seems probable therefore that the progression of prostate cancer in TRAMP is IGF-I dependent.

The IGF axis is an important regulator of growth and development

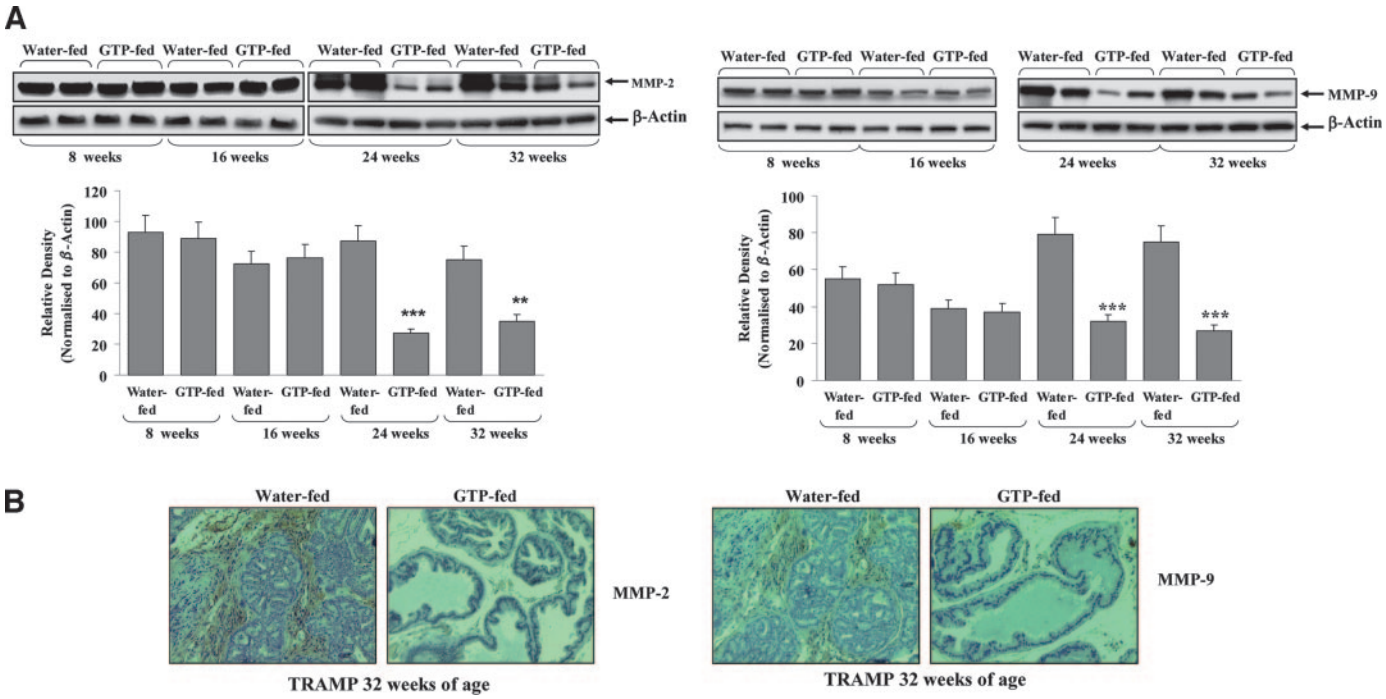

Fig. 4. Effect of green tea polyphenol infusion on MMP-2 and MMP-9 protein levels in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, protein levels of MMP-2 and MMP-9 by immunoblot analyses. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Western blot analysis was conducted in five animals in each group, and only representative blots for two animals are shown. Histogram represents relative density data of the immunoblots in relative units ± *SEM*. Values represent mean ± *SEM*. **, $P < 0.01$; ***, $P < 0.001$ compared with water-fed control groups. *A*, protein levels of MMP-2 and MMP-9 by immunohistochemical analyses. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Representative photomicrographs (magnification, ×40) of immunohistochemical staining for MMP-2 in water-fed control and green tea polyphenol-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data were confirmed in two slides from five animals.

and changes in IGF signaling have important implications in malignant growth of prostate cancer (19–22, 35–37). Although primarily synthesized in the liver, IGFs are produced locally by most tissues in which they act in an autocrine or paracrine manner (35–37). Deregulation of the IGF axis and elevated serum levels of IGF-I are associated with prostate cancer (20, 21, 36, 38). IGF-I binds to the IGF-I receptor, a tyrosine kinase receptor that transduces signals to the nucleus and mitochondrion primarily via the MAPK and PI3k/Akt pathways (29–30). In addition to direct contributions to each of these stages, IGF-I may promote cancer indirectly, through interactions with oncogenes and tumor suppressors, interactions with other hormones, and interactions with the IGF binding proteins (21, 29, 37). Prompted by the accumulating evidence, investigations are also being pursued to modulate the IGF system as a possible means of cancer prevention or treatment (8, 38, 39). In the TRAMP mice, IGF-I has been implicated as an important factor in the development and progression of prostate cancer (8, 20, 40). Elevated IGF-I levels in the TRAMP model have also been proposed to induce the expression of VEGF, thereby facilitating angiogenesis and leading to metastatic spread of the disease (20). This model possesses similarity to the human disease in the development and progression of metastatic prostate cancer, and the utility of this model in chemoprevention studies has been demonstrated by studies from our laboratory and elsewhere (8, 26, 40–44).

A potential consequence of increased IGF-I is the activation of multiple signal transduction pathways including the MAPK pathway implicated in mitogenesis and the PI3k-dependent pathway implicated in the transmission of cell survival signals (28–30). Examining the expression of these proteins revealed increasing levels of the catalytic subunit of PI3k. Furthermore, progression of prostate cancer in TRAMP was accompanied by increased phosphorylation of Akt/PKB. Activation of the catalytic subunit of PI3k results in the production of phosphatidyl inositols and causes membrane translocation, phosphorylation, and activation of the Ser/Thr kinase Akt/PKB, a major transducer of the PI3k signal (45). Our data further indicated the involvement of ERK in the transduction of IGF-I–induced signaling in TRAMP. Although IGF-I–induced signaling is known to involve either of the PI3k or ERK signaling (based on cell type), we observed activation of both of these pathways in our study. This could be explained on the basis of the fact that prostate cancer tumors represent a mixture of different cell types each using its own distinct signaling pathway. Because the protein expression of pERK was greater than that of pAkt (Fig. 2), it could be speculated that IGF-I–induced signaling in the TRAMP predominantly involves the ERK pathway.

Our studies demonstrated that oral infusion of green tea polyphenols resulted in significant inhibition of prostatic IGF-I and restoration of IGFBP-3 levels. This was accompanied with inhibition of downstream signaling cascade that involved both the PI3k/Akt and the MAPK (ERK) signaling pathways. This observation bears significance in light of the studies that indicate increased levels of IGF-I are associated with increased risk of several cancers such as those of breast, prostate, lung, and colon and bears credence to the fact that inhibition of the IGF axis could be a potential mechanism for prevention of prostate cancer (35).

In the TRAMP model, serum IGF-I levels correlate with the increase in mean vessel density associated with the development of high-grade PIN lesions, suggesting a relationship between IGF-I and the induction of prostatic neovascularization (20). We therefore investigated modulation of various markers of angiogenesis and metastasis by oral infusion of green tea polyphenol. Our earlier observations had suggested inhibition of invasion and metastasis by green tea polyphenols in TRAMP (8). In this study, green tea polyphenol

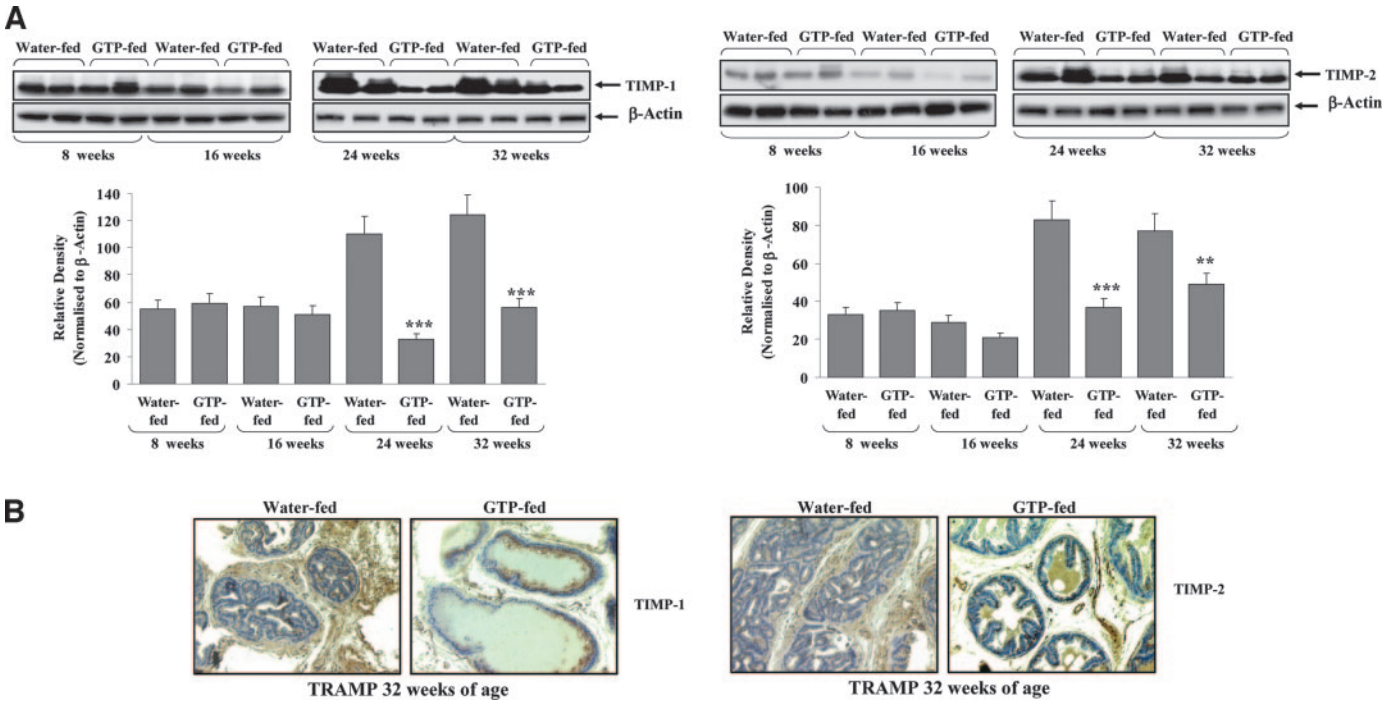

Fig. 5. Effect of green tea polyphenol infusion on TIMP-1 and TIMP-2 protein levels in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, protein levels of TIMP-1 and TIMP-2 by immunoblot analyses. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Western blot analysis was conducted in five animals in each group, and only representative blots for two animals are shown. Histogram represents relative density data of the immunoblots in relative units ± *SEM*. Values represent mean ± *SEM*. **, $P < 0.01$; ***, $P < 0.001$ compared with water-fed control groups. *B*, protein levels of TIMP-1 and TIMP-2 by immunohistochemical analyses. As detailed in Materials and Methods, the protein levels were determined in the dorso-lateral prostate of water-fed control and green tea polyphenol-fed TRAMP mice at 16, 24, and 32 weeks of age. Representative photomicrographs (magnification, ×40) of immunohistochemical staining for MMP-2 in water-fed control and green tea polyphenol-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data were confirmed in two slides from five animals.

infusion in TRAMP mice resulted in significant inhibition of VEGF, a known marker of angiogenesis. This inhibition correlated with the time period in the TRAMP mice when prostatic neovascularization the "angiogenic switch" begins to set in (20). Inhibition of VEGF by green tea has previously been investigated in a mouse model of corneal neovascularization (46). In this model, drinking green tea (1.25% in drinking water) was associated with significant inhibition of VEGF-induced corneal neovascularization. Because the growth of all solid tumors is dependent on angiogenesis, inhibition of VEGF by green tea could explain why drinking green tea prevents the growth of a variety of tumors (46).

Because all cancers need proteolytic enzymes to invade surrounding tissue and metastasize, we next investigated the inhibitory effect of green tea polyphenol infusion on several markers of metastasis in TRAMP mice. We observed that protein expression and activity of uPA was significantly inhibited by oral infusion of green tea. Although inhibition of protein expression of uPA was evident at 24 and 32 weeks of age, inhibitions of the activity of uPA were detected as early as 8 weeks and continued throughout the 24-week period under test. This observation assumes significance because it has been observed that EGCG, the major ingredient of green tea, directly binds to the catalytic triad of uPA and interferes with the ability of uPA to recognize its substrates and inhibit enzyme activity. Such inhibitory effects are likely to reduce the size of tumors or inhibit the progression of cancer.

Several studies have shown a close relationship between the expression of members of MMP family by tumors and their metastatic potential (33, 34 and references therein). Matrix metalloproteinases, in particular MMP-2 and MMP-9, are a family of secreted and membrane bound zinc-endopeptidases under the inhibitory control of TIMP-2 and TIMP-1, respectively, thus maintaining a balance between matrix degradation and formation (34). Oral infusion of green tea polyphenols in TRAMP was associated with inhibition of both MMP-2 and MMP-9 in the dorso-lateral prostate. This inhibition was observed at 24 and 32 weeks of age, coinciding with the time points

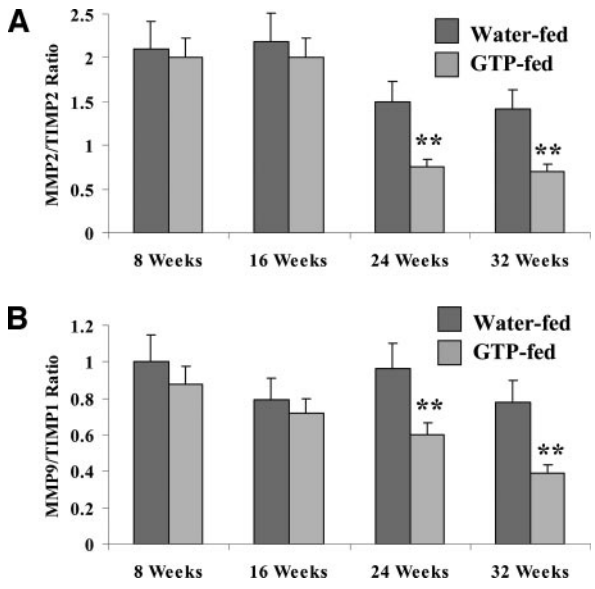

Fig. 6. Effect of green tea polyphenol infusion on the ratio of MMP to TIMP in the dorso-lateral prostate during progressive stages of prostate cancer development in TRAMP mice. *A*, effect of green tea polyphenol infusion on MMP2/TIMP2 ratio. *B*, effect of green tea polyphenol infusion on MMP9 to TIMP1 ratio. The ratios were determined from the data obtained by immunoblot analyses of MMPs and TIMPs. Data are means ± *SEM* of five animals. **, $P < 0.01$ compared with respective age-matched water-fed TRAMP mice.

at which metastatic spread of the disease is observed in this model. Although apparently an inhibition in the expression of TIMP-1 and TIMP-2 was observed with green tea polyphenol, an estimation of the ratios of MMP to TIMP revealed that in green tea polyphenol-fed TRAMP mice, the balance between MMP and TIMP significantly favored TIMP levels. The balance between MMP-9 and MMP-2 to TIMP-1 and TIMP-2 expression was shown to be an essential factor in the aggressiveness of several cancers (47, 48). In this study, the ratio of MMP to TIMP was greater than 1 in water-fed TRAMP mice and less than 1 in green tea polyphenol-fed TRAMP mice. Several studies have observed inhibition in the expression of MMPs by green tea (47–50), and tumor cell invasion of a reconstituted basement membrane matrix was reduced by 50% with EGCG a green tea component at concentrations equivalent to that in the plasma of moderate green tea drinker (50).

In conclusion, our results suggest that increased IGF-I signaling in TRAMP may induce tumor development and its progression and that green tea polyphenol, a polyphenolic mixture, inhibits the IGF-I–induced signaling thereby inhibiting the progression and invasion of prostate cancer. The knowledge of the interactions between dietary agents and molecules of the matrix degradation is being pursued in earnest in an attempt to devise nontoxic dietary agents for cancer chemoprevention. These results indicate a role for green tea in the prevention of prostate cancer.

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