Mechanism of Cancer Chemoprevention by Tea Polyphenols in TRAMP Mice: Involvement of Insulin-Like Growth Factor-(IGF)-1 Signaling
Chapter IV

Chapter 4

Mechanism of Cancer Chemoprevention by Tea Polyphenols in TRAMP Mice: Involvement of Insulin-Like Growth Factor-(IGF)-I Signaling

Studies described in this chapter have investigated the involvement of IGF-I signaling in green tea polyphenols (GTP)-mediated suppression of prostate Cancer (CaP) progression, metastasis and angiogenesis in an autochthonous mouse model of prostate cancer viz. transgenic adenocarcinoma of the mouse prostate (TRAMP). A detail description of the study is as follows:

4.1 Introduction and Rationale

The polyphenols obtained from green tea (GTP) are potential chemopreventive agents against prostate cancer (CaP), primarily because of their high intake by populations with reduced cancer incidence and their reported ability to inhibit proliferation and induction of apoptosis in CaP cells in culture (Klein and Thompson, 2004; Adhami et al., 2003; Ahmad et al., 1997; Gupta et al., 1999; Gupta et al., 2000; Saleem et al., 2003; Siddiqui et al., 2004a; Park & Surh, 2004; Brusselmans et al., 2003). It was earlier shown that oral infusion of GTP to 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 CaP and its subsequent progression (Gupta et al., 2001a). An important observation from this study was the inhibition of serum insulin-like growth factor (IGF-I) levels with concomitant increase in insulin-like growth factor binding protein-3 (IGFBP-3) levels in GTP-fed mice. This is consistent with observations in humans where most case control studies have shown increased IGF-I levels in men with CaP compared to men with benign prostatic hyperplasia (BPH) or controls (Wolk et al., 1998; Chan et al., 1998; Woodson et al., 2003; Chan et al., 2002; Kaplan et al., 1999). The increased levels of IGF could also contribute to the initiation and progression of CaP since 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 neo-vascularization (Kaplan et al., 1999). It is known that type I IGF receptor is a regulator of matrix metalloproteinase (MMP)-2 synthesis (Grzmil et al., 2004). Increased IGF signaling also enhances the expression of VEGF, urokinase plasminogen activator (uPA) and MMPs, which in turn closely correlate with tumor angiogenesis and metastasis (Kaplan et al., 1999; Grzmil et al., 2004; Stattin et al., 2001; Zhang et al., 2003; Martin et al., 1999; Bergers et al., 2000). Understanding the molecular mechanisms of GTP mediated inhibition of CaP is essential in devising rationale and mechanism based chemopreventive approaches.

In this chapter, studies are presented showing that GTP-mediated suppression of CaP progression, metastasis and angiogenesis may be mediated through inhibition of IGF and its down stream signaling pathway and suggest that IGF/IGFBP-3 pathway as a target for GTP-mediated inhibition of CaP progression in TRAMP models.

4.2 Materials and Methods

Animals

The male and female heterozygous C57BL/1TGN TRAMP mice, Line PB Tag 8247NG were purchased as breeding pairs from Jackson Laboratory (Bar Harbor, Maine). The animals were bred on same genetic background and maintained in the Animal Care Facility of Case Western University or University of Wisconsin. Transgenic males for these studies were routinely obtained as [TRAMP X C57BL/6] F1 or as [TRAMP x C57BL/6] F2 offspring. Identity of transgenic mice was established by the PCR-based DNA-screening as previously described (Greenberg et al., 1995). At or before 4 weeks of age mice were prepared for obtaining tail snips, the tails were disinfected by washing with an alcohol swab. One centimeter of the tail end were cut using clean, sterilized and sharp scalpers or scissors. The cut tail end then held tightly for one minute with a dry swab to minimize bleeding. Cut tail ends were used for extraction of DNA and identification of the transgene by PCR amplification. The transgenic male animals obtained were employed in the study.
Study Design and Green Tea Polyphenol Supplementation

Green tea polyphenols (GTP >95% enriched preparation) was 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 access to laboratory chow ad libitum. A freshly prepared solution of 0.1% GTP in tap-water was supplied every Monday, Wednesday and Friday to experimental animals as the sole source of drinking fluid for 24 weeks (GTP-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 animals and has been used in mice in many previous studies from various laboratories and is equivalent to an approximate consumption of six cups of green tea per day by an average adult human (Gupta et al., 2001a and the references therein). For each experiment, 20 male TRAMP mice, 8 weeks of age were divided into two equal groups consisting of 10 animals in each group. At each time point and at the termination of the experiment the mice were killed by cervical dislocation and the dorsolateral prostate was removed under a dissecting microscope for biochemical and histological analysis.

Preparation and Analysis of Tissue

The dorso-lateral prostate was excised, 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. Histological sections were reviewed by light microscopy for the presence of CaP 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) CaP, 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 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na$_3$VO$_4$, 0.5% NP40, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, pH 7.4) at 4°C. The homogenates were centrifuged at 14000xg for 20 minutes and the supernatant (total cell lysate) were collected. The protein concentration was determined and western blot analysis was performed as mentioned in Chapter III. The blots were blocked using 5% non-fat dry milk and probed using appropriate primary antibody of IGF-I, IGFBP-3 (Cell Signaling Technology, Beverly, MA), VEGF, uPA (Santa Cruz Biotechnology, Santa Cruz, CA), PI3K, Akt, Erk1/2 (Transduction Laboratories, VA), β-Actin (Sigma, St Louis, MO) and MMP-2, MMP-9, TIMP-1 and TIMP-2 (Chemicon International, Temecula, CA) in blocking buffer overnight at 4°C.

Immunobots 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 ± SE followed by appropriate statistical analysis.

Immunohistochemical Analysis

Sections (4 µm) were cut from paraffin-embedded prostate tissues obtained from TRAMP mice following treatments. 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 (Gupta et al., 2004). The stained slides were dehydrated and mounted in permount and visualized on a Zeiss-Axiophot DM HT microscope (Zeiss-Axiophot, 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 protocol described earlier (Heussen and Dowdle, 1980). Fifty μg of the prostate tissue lysate was dissolved in SDS sample buffer and run under non-denaturing 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 min in buffer containing 5 mM calcium chloride, 1 μM zinc chloride, 50 mM 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 M NaCl, 0.02% sodium azide and 10 mM 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 VEGF

A sandwich enzyme immuno assay technique (Mouse VEGF Immuno assay, R & D Systems, Minneapolis, MN) was utilized to quantitate VEGF levels in the serum of TRAMP mice by following the manufacturer’s protocol. The assay employs the quantitative sandwich enzyme immunoassay technique. Affinity purified polyclonal antibody specific for mouse VEGF is pre-coated onto the microplate. Samples were pipetted into the wells and any VEGF present gets bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is proportional to the amount the amount of mouse VEGF bound in the initial step. The sample values are then read off the standard curve. 50 μl of Assay diluent (buffered protein solution with preservatives) is added to each well followed by 50 μl of standard or sample in each well. It is mixed gently by tapping for 1-2 minutes and incubated for 2 hours at RT. The wells were aspirated and washed with 400 μl of wash buffer (buffered surfactant with
preservatives) for a total of five washes. 100 µl of mouse VEGF conjugate is then added to the wells and incubated for another 2 hours and washing is repeated as before. 100 µl of substrate solution (equal mixture of stabilized hydrogen peroxide and tetramethylbenzidine) is added and incubated at RT for 30 minutes protected from light followed by adding 100 µl of stop solution (diluted hydrochloric acid solution) and mixed thoroughly. The optical density is measured at 450 nm with a wavelength correction at 540 nm and results were represented as VEGF levels in pg/ml 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 GTP comparisons were made with age-matched water-fed TRAMP mice. Values of p<0.05 were considered statistically significant.

4.3 Results

Studies have shown an increase in serum IGF-I levels with concomitant decrease in serum IGFBP-3 levels in TRAMP mice during CaP progression (Gupta et al., 2001a). Since IGFs are known to be produced locally by most tissues, in which they act in an autocrine or a paracrine manner (Djavan et al., 1999; Shi et al., 2001; Grimberg, 2003), this study further determined IGF-1 and IGFBP-3 expression in the dorsolateral 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. 1A). 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. 1A).
Figure 1: Expression of IGF-I and IGFBP-3 in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A). Protein expression analysis of IGF-I and IGFBP-3 by immunoblotting in the dorsolateral prostate of 8, 16, 24 and 32 weeks old TRAMP mice. Equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. B). Histogram indicates the ratio between IGF-I and IGFBP-3 obtained by densitometric analysis of the bands shown in A normalized to β-actin. C). Photomicrographs (Magnification, 40X) represent immunohistochemical staining for IGF-I and IGFBP-3 in TRAMP with moderately differentiated (24 weeks) poorly differentiated (32 weeks) adenocarcinoma. Western blot analysis was conducted in 5 animals in each group and only representative blots are shown. Immunostaining data was confirmed in two slides from five animals.
Densitometric analysis revealed that the progression of cancer in TRAMP mice was associated with a shift in IGF-I/IGFBP-3 ratio favoring cancer progression (Fig. 1B). Similar results were observed by immuno-histochemical analysis of the prostate tissue of TRAMP mice (Fig. 1C). 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. 1C).

Intrinsic induction of IGF-I can trigger multiple signal transduction pathways that include the MAP kinase (ERK) pathway and the PI3 kinase dependent pathway implicated in the cell survival signals (Grimberg, 2003; Zhang and Brodt; 2003; Song et al., 2003). This study, therefore, analyzed the expression of PI3K, Akt and ERK1/2 in the dorsolateral 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 PI3K was observed (Fig. 2A). Further, downstream of PI3 kinase, we observed, increased phosphorylation of Akt at Thr 308 (Fig. 2B) and ERK1/2 (Fig. 2C) without any change in the expression of total Akt and ERK expression in the dorso-lateral prostate of TRAMP mice (Fig. 2B & 2C). When the data was analyzed densitometrically we observed that the increase in the expression of these proteins was significantly higher (p<0.05) compared to animals of the preceding age (Fig 2A, B and C).

It has been previously observed that GTP infusion to TRAMP mice significantly lowered serum IGF-I and restored serum IGFBP-3 levels (Gupta et al., 2001a). As shown in Figure 3A continuous GTP infusion to TRAMP mice resulted in significant inhibition in the protein expression of IGF-I with concomitant restoration of IGFBP-3 levels in the dorsolateral prostate tissue of TRAMP. Densitometric analysis of the bands suggested that IGF-I/IGFBP-3 ratios were inhibited by 70% to 83% (p<0.01) by oral feeding of GTP (Fig. 3A). These results were further confirmed by immunohistochemical analysis of IGF-I levels (Fig. 3B) indicating a significant decrease in IGF-I expression in GTP-fed
TRAMP mice. PI3K levels were significantly lowered by oral infusion of GTP (by 67% to 79%, p<0.01, Fig. 4A) and phosphorylation of Akt at Thr308 was inhibited in GTP-fed TRAMP mice (upto 65%, p<0.01, Fig. 4B). We also observed significant inhibition (50% to 62%, p<0.01) in the phosphorylation of ERK1/2 (Fig. 5) in TRAMP mice that received GTP as the sole source of drinking water.

One possible consequence of increased IGF-I levels is the promotion of angiogenesis since it is known that IGF-I can induce VEGF (Poulaki et al., 2003; Turner et al., 2003). Therefore, we determined the effect of GTP infusion on VEGF in TRAMP mice. We observed decreased protein expression of VEGF in the prostate of GTP-fed mice compared to the control water-fed group (Fig. 6A). 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. 6B). While 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 GTP-fed TRAMP mice (by 43% at 24 weeks, p<0.01 and 71% at 32 weeks, p<0.001, Fig. 6C).

We next determined the effect of GTP infusion on the urokinase-type plasminogen activator (uPA) which is involved in the metastasis of several cancers (de Bock and Wang, 2004; Mazar et al., 1999). As shown in Figure 7A protein levels of uPA was significantly higher in GTP-fed TRAMP mice with moderately differentiated (24 weeks) and poorly differentiated (32 weeks) adenocarcinoma. GTP infusion 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. 7B). Interestingly, gelatin zymography revealed that the activity of uPA was inhibited by GTP at all time points (Fig. 7C). While 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 was observed at all time points (Fig. 7D).
Figure 2: Protein expression of PI3K, Akt and ERK1/2 in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: Western blot analysis for protein expression of A) PI3K, B) phosphoAkt, C) phosphoErk1/2 in the dorsolateral prostate of 8, 16, 24 and 32 weeks 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 5 animals in each group and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units ± SE. ** indicates p<0.01 and *** indicates p<0.001 compared with water-fed TRAMP mice of the preceding age.
Figure 3: Effect of GTP infusion on tissue IGF-I, IGFBP-3 expressions in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: Western blot analysis for protein expression of A) IGF-I, IGFBP-3 in the prostate of water-fed control and GTP-fed TRAMP mice at 16, 24 and 32 weeks of age. Blots were stripped and reprobed for β-actin, total Akt and total Erk to confirm equal loading of the protein. Western blot analysis was conducted in 5 animals in each group and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units ± SE. * indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001 compared with respective age-matched water-fed TRAMP mice. B) Representative photomicrographs (Magnification, 40X) of immunohistochemical staining for IGF-I in water-fed control and GTP-fed TRAMP mice with moderately differentiated (24 weeks) poorly differentiated (32 weeks) adenocarcinoma. Immunostaining data was confirmed in two slides from five animals.
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Figure 4: Effect of GTP infusion on tissue PI3K and Akt expressions during progressive stages of CaP development in TRAMP mice: Western blot analysis for protein expression of C) PI3K, D) phosphoAkt in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 16, 24 and 32 weeks of age. Blots were stripped and reprobed for analysis of β-actin and total Akt to confirm no change in the protein. Western blot analysis was conducted in 5 animals in each group and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units ± SE. * indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001 compared with respective age-matched water-fed TRAMP mice.
Figure 5: Effect of GTP infusion on tissue ERK expressions during progressive stages of CaP development in TRAMP mice: Western blot analysis for protein expression of phosphoErk1/2 in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 16, 24 and 32 weeks of age. Blots were stripped and reprobed for analysis of total Erk to confirm no change in the protein. Western blot analysis was conducted in 5 animals in each group and only representative blots are shown. Histogram represents relative density data of the immunoblots in relative units ± SE. * indicates p<0.05, ** indicates p<0.01 and *** indicates p<0.001 compared with respective age-matched water-fed TRAMP mice.
The matrix metalloproteinase (MMP) are known plays a key role in matrix degradation thus allowing tumor dissemination (Freije et al., 2003 and references therein). Cells with invasive potential are known to secrete active forms of MMP-2 and MMP-9 in a process that is inhibited by tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 and TIMP-2 (Hojilla et al., 2003; Hidalgo and Eckhardt, 2001). In the next series of experiments, we determined the tissue levels of MMPs and the TIMPs in control and GTP-fed TRAMP mice. We observed increased protein expression of MMP-2 (Fig. 8A) and MMP-9 (Fig. 9A) particularly in mice with moderately and poorly differentiated adenocarcinoma, time points that coincide with the initiation of metastasis. This increased expression was significantly inhibited (by 53% to 68%, p<0.01) by continuous GTP 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% GTP in drinking water (Figs. 8B and 9B). Similar results were observed by immunohistochemical analysis where significant decrease in the protein expression of MMP-2 (Fig. 8C) and MMP-9 (Fig. 9C) was observed in mice with poorly differentiated adenocarcinoma (32 weeks) after GTP feeding.

Endogenous levels of tissue inhibitors of metalloproteinases (TIMP) specifically inhibit the matrix metalloproteinases (Hojilla et al., 2003). The expression of MMPs and TIMPs has been proposed to be co-regulated and an imbalance between them has been shown to be an essential factor in the invasive phenotype of cancers (Hidalgo and Eckhardt, 2001). An increase in the TIMP expression was observed in TRAMP mice similar to that of MMP expression. Continuous GTP-infusion to TRAMP mice resulted in inhibition of TIMP expression in mice with moderately (24 weeks, p<0.01) and poorly (32 weeks, p<0.01) differentiated adenocarcinoma (Figs. 10A, 10B, 11A & 11B). These observations were further analyzed and confirmed by immunohistochemistry of TIMP-1 and TIMP-2 (Figs. 10C & 11C) in the prostate tissues of water-fed TRAMP and GTP-fed TRAMP animals. Interestingly, analysis of MMP to TIMP ratio in TRAMP mice indicates that the balance was shifted in such a way that favored MMP expression whereas continuous GTP
**Figure 6:** Effect of GTP infusion on VEGF expression and levels in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of eVEGF in the dorsolateral prostate of control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. B) Histogram represents relative density data of the immunoblots in relative units ± SE. ** indicates \( p<0.01 \) and *** indicates \( p<0.001 \) compared with respective age-matched water-fed TRAMP mice. Representative data from 5 animals in each group are shown. C) Enzyme linked immunosorbant assay for quantitative evaluation of VEGF levels in the serum of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. For VEGF analysis 5 samples of each group in triplicate were analyzed. Values represent mean ± SE and * indicates **\( p<0.01 \), *** indicates \( p<0.001 \) compared with respective water-fed control groups.
Figure 7: Effect of GTP infusion on urokinase plasminogen activator (uPA) expression and activity in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of uPA in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks. B) Histogram represents relative density data of the immunoblots in relative units ± SE of the bands in A. Representative data from 5 animals in each group are shown. * indicates p<0.05 and ** indicates p<0.01 compared with control TRAMP mice C). Gelatinolytic activity of uPA as analyzed by gelatin zymography. D) Histogram represents relative density of the zymogram in relative units ± SE. Values represent mean ± SE. * indicates p<0.05, *** indicates p<0.001 compared with respective water-fed control group.
Figure 8: Effect of GTP infusion on matrix metalloproteinase (MMP)-2 expression in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of MMP-2 in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. Representative data from 5 animals in each group are shown. B) Histogram represents relative density data of the immunoblots in relative units ± SE. Values represent mean ± SE. ** indicates p<0.01 and *** indicates p<0.001 compared with water-fed control groups. C) Representative photomicrographs (Magnification, 40X) of immunohisto-chemical staining for MMP-2 in water-fed control and GTP-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data is a representative from five animals.
Figure 9: Effect of GTP infusion on matrix metalloproteinase (MMP)-9 expression in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of MMP-9 in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. Representative data from 5 animals in each group are shown. B) Histogram represents relative density data of the immunoblots in relative units ± SE. *** indicates p<0.01 compared with water-fed TRAMP mice. B). C) Representative photomicrographs (Magnification, 40X) of immunohistochemical staining for MMP-9 in water-fed control TRAMP and GTP-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data is a representative from five animals.
Figure 10: Effect of GTP infusion on tissue inhibitor of matrix metalloproteinase (TIMP)-1 expression in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of TIMP-1 in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. Representative data from 5 animals in each group are shown. B) Histogram represents relative density data of the immunoblots in relative units ± SE. *** indicates p<0.001 compared with respective age-matched water-fed TRAMP mice. C) Representative photomicrographs (Magnification, 40X) of immuno-histochemical staining for TIMP-1 in water-fed control TRAMP and GTP-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data is a representative from five animals.
Figure 11: Effect of GTP infusion on tissue inhibitor of matrix metalloproteinase (TIMP)-2 expression in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: A) Western blot analysis of TIMP-2 in the dorsolateral prostate of water-fed control and GTP-fed TRAMP mice at 8, 16, 24 and 32 weeks of age. Representative data from 5 animals in each group are shown. B) Histogram represents relative density data of the immunoblots in relative units ± SE. ** indicates p<0.01 and *** indicates p<0.001 compared with respective age-matched water-fed TRAMP mice. C). Representative photomicrographs (Magnification, 40X) of immunohistochemical staining for TIMP-2 in water-fed control TRAMP and GTP-fed TRAMP with poorly differentiated adenocarcinoma (32 weeks of age). Immunostaining data is a representative from five animals.
Figure 12: Effect of GTP infusion on the ratio of MMP/TIMP in the dorsolateral prostate during progressive stages of CaP development in TRAMP mice: Histograms represent ratios of A) MMP2/TIMP2 and B) MMP9/TIMP1 calculated from the relative densities of each protein. Data are mean ± SE of five animals. ** indicates p<0.01 compared to respective age-matched water-fed TRAMP mice.
infusion to TRAMP mice favored this ratio towards TIMP expression suggesting inhibition (p<0.01) of MMP expression (Fig. 12A & B)

4.4 Discussion

The limited available options for the treatment of CaP have prompted the need for developing alternative strategies for management of CaP. Chemoprevention by the use of dietary or non-toxic synthetic agents has offered a viable option to block neoplastic inception or delay disease progression. Because CaP is typically diagnosed in men aged 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. Studies have shown that oral infusion of a human achievable dose of green tea results in significant inhibition in the development and progression of CaP along with increased survival in TRAMP (Gupta et al., 2001a). This study examines the underlying mechanisms to understand if IGF-I induced signaling pathways are modulated by oral infusion of green tea polyphenols and further determines if this feeding regimen inhibits the expression of molecules involved in metastasis and angiogenesis.

To begin with the study evaluates levels of IGF-I and its binding protein IGFBP-3 in the dorsolateral prostate of TRAMP mice at ages that were pathologically distinct from each other. We observed that as cancer progressed from no 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 where prostate-specific IGF-I was found to be increased during CaP progression in TRAMP mice (Kaplan et al., 1999). This increase in serum IGF-I is probably due to prostatic response rather than due to a systemic response (Kaplan et al., 1999). It seems probable therefore that the progression of CaP in TRAMP is IGF-I dependent.
The IGF axis is an important regulator of growth and development and changes in IGF-signaling have important implications in malignant growth of CaP (Chan et al., 2002; Kaplan et al., 1999; Grzmil et al., 2004; Stattin et al., 2001; Djavan et al., 2001; DiGiovanni et al., 2000; Signorello et al., 1999; Peehl et al., 1996). Although primarily synthesized in the liver, IGFs are produced locally by most tissues where they act in an autocrine or paracrine manner (DiGiovanni et al., 2000; Signorello et al., 1999; Peehl et al., 1996). Deregulation of the IGF axis and elevated serum levels of IGF-I are associated with CaP (Kaplan, et al., 1999; Grzmil et al., 2004; DiGiovanni et al., 2000; Burfeind et al., 1996). IGF-I binds to the IGF-I receptor, a tyrosine kinase receptor that transduces signals to the nucleus and mitochondrion primarily via the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways (Grimberg, 2003; Zhang and Brodt, 2003; Song et al., 2003). 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 (Grzmil et al., 2004; Grimberg, 2003 and Cohen et al., 1994). Prompted by the accumulating evidence, investigations are also being pursued to modulate the IGF system as a possible means of cancer prevention or treatment (Gupta et al., 2001a; Martin et al., 1999; Burfeind et al., 1996; Wang et al., 2003). In the TRAMP mice IGF-I has been implicated as an important factor in the development and progression of CaP (Gupta et al., 2001a; Kaplan et al., 1999; Gingrich et al., 1997). 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 (Kaplan et al., 1999). This model possesses similarity to the human disease in the development and progression of metastatic CaP and the utility of this model in chemoprevention studies have been demonstrated by various studies (Gupta et al., 2001a; Gupta et al., 2004; Gingrich et al., 1997; Huss et al., 2001; Gupta et al., 2001b; Raghow et al., 2002; Mentor-Marcel et al., 2001).

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 (Shi et al., 2001; Grimberg, 2003; Zhang et al., 2003; Song
et al., 2003; Bhattacharya and Stern, 2003). Examining the expression of these proteins revealed increasing levels of the catalytic subunit of PI3K. Further, progression of CaP in TRAMP was accompanied by increased phosphorylation of Akt/PKB. Activation of the catalytic subunit of PI3K results in the production of phosphatidylinositols and causes membrane translocation, phosphorylation and activation of the serine/threonine kinase Akt/PKB, a major transducer of the PI3K signal (Zhang and Brodt, 2003; Siddiqui et al., 2004b). The data further indicated the involvement of ERK in the transduction of IGF-I induced signaling in TRAMP. Although IGF-I induced signal transduction involves either PI3K or the ERK signaling being cell-context dependent we observed activation of both pathways. This could be explained on the basis of the fact that CaP tumors represent a mixture of different cell types each utilizing its own distinct signaling pathway. Since 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.

The study demonstrated that oral infusion of GTP resulted in significant inhibition of prostatic IGF-I and restoration of IGFBP-3 levels. This was accompanied with inhibition of down stream 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 CaP (Djavan et al., 2001; DiGiovanni et al., 2000).

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 (Kaplan et al., 1999). This chapter therefore investigates modulation of various markers of angiogenesis and metastasis by oral infusion of GTP. Earlier observations had suggested inhibition of invasion and metastasis by GTP in TRAMP (Gupta et al., 2001a & b). In this study GTP 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 (Kaplan et al., 1999). Inhibition of VEGF by green tea has previously been investigated in a mouse model of corneal neovascularization (Cao and Cao, 1999). 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 (Cao and Cao, 1999).

Since all cancers need proteolytic enzymes to invade surrounding tissue and metastasize, this chapter next investigates the inhibitory effect of GTP infusion on several markers of metastasis in TRAMP mice and illustrates that protein expression and activity of uPA was significantly inhibited by oral infusion of green tea. While 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 since it has been observed that EGCG, the major ingredient of green tea directly binds to the catalytic triad of uPA and interfering 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 (Freije et al., 2003; Hojilla et al., 2003; Hidalgo and Eckhardt, 2001 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 (Hojilla et al., 2003). Oral infusion of GTP in TRAMP was associated with inhibition of both MMP-2 and MMP-9 in the dorsolateral prostate. This inhibition was observed at 24 and 32 weeks of age coinciding with the time points 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 GTP, an estimation of the ratios of MMP/TIMP revealed that
in GTP-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 (Oku et al., 2003; Freije et al., 2003; Hojilla et al., 2003). In this study the ration of MMP/TIMP was greater than 1 in water fed TRAMP mice and less than 1 in GTP-fed TRAMP mice. Several studies have observed inhibition in the expression of MMPs by green tea (Oku et al., 2003; Annabi et al., 2003; Demeule et al., 2000; Pezzato et al., 2003; Garbisa et al., 2001) 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 (Garbisa et al., 2001).

In conclusion, the results suggest that increased IGF-I signaling in TRAMP may induce tumor development and its progression and that GTP, a polyphenolic mixture, inhibits the IGF-I induced signaling thereby inhibiting the progression and invasion of CaP.

References


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Chapter IV


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