Chapter V

Prostate Cancer Chemoprevention by Tea Polyphenols: Studies in Nude Mice
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Chapter 5

Prostate Cancer Chemoprevention by Tea Polyphenols: Studies in Nude Mice

Studies described in this chapter have investigated the cancer chemopreventive as well as cancer therapeutic potential of tea polyphenols against prostate cancer in athymic nude mice implanted with androgen-responsive CWR22Rv1 or androgen-unresponsive PC-3 prostate carcinoma cells. A detailed description of the study is as follows:

5.1 Introduction and Rationale

Studies have established that regulation of the development and function of prostate is intimately associated with androgen action and prostate cancer is known to undergo a transition from an early 'androgen-sensitive' form of cancer to a late (metastatic) 'androgen-insensitive' cancer, and at the time of clinical diagnosis most prostate cancers (CaPs) represent a mixture of androgen-sensitive and androgen-insensitive cells. Published studies from this laboratory as well as from other laboratories have shown that polyphenols present in tea plant Camelia sinensis possess CaP chemopreventive properties (Gupta et al., 2001; Ahmad et al., 1997; Baatout et al., 2004; Caporali et al., 2004; Chung et al., 2001; Adhami et al., 2003a; Siddiqui et al., 2004). The mechanism of these remarkable cancer chemopreventive effects of tea polyphenols is not completely understood. Our studies presented in chapter II have shown that tea polyphenols (aqueous extract of black tea) inhibit androgen-mediated oxidative injury in the prostate of Wistar rats. In chapter III of this thesis, we demonstrated that a modulation of PI3K/Akt and Erk1/2 pathways by EGCG as well as TF in cell culture system. In chapter IV, we demonstrated that GTP-mediated suppression of CaP progression, metastasis and angiogenesis may be mediated through inhibition of IGF and its down stream signaling events including MMPs, TIMPs and VEGF; and suggested that IGF/IGFBP-3 pathway as a target for GTP-mediated inhibition of CaP progression in TRAMP model. Other studies have suggested the involvement of multiple pathways during CaP chemopreventive effects of tea polyphenols. Hussain et al. (2004) showed that EGCG inhibits
COX-2 without affecting COX-1 expression at both the mRNA and protein levels, in androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate carcinoma cells. Caporali et al. demonstrated an involvement of clusterin, a protein involved in many different processes including apoptosis and neoplastic transformation, during CaP chemopreventive effects of green tea catechins in TRAMP model (Caporali et al., 2004). Recently, Pezzato et al. (2004) demonstrated that EGCG inhibited PSA expression and activities in prostate carcinoma cells. This study also demonstrated that PSA causes a degradation of type IV collagen in reconstituted basement membrane (Matrigel) and activation of progelatinase A (MMP-2), but not pro-MMP-9, in a cell-free system (Pezzato et al., 2004). In another recent study Sartor et al. (2004) demonstrated that (i) EGCG down-modulated a biochemical mechanism instrumental to invasion and metastasis, MMP-2 expression, and inhibit tumor cell chemo-invasion through reconstituted basement membrane, (ii) green tea abolished tumor growth containment triggered by inflammatory cell recruitment, and (iii) EGCG inhibited PMN-triggered activation of tumor MMP-2. According to the authors, the first point (i) gives a biochemical explanation to the inhibition of metastatic aggressiveness of CaP in the same model system. The other 2 points (ii, iii) suggest that an acute inflammatory involvement of CaP could be efficaciously prevented by green-tea consumption, in association with a concomitant lowering of the invasive potential.

However, till date, no study has compared the CaP chemopreventive effects of polyphenols from green tea versus black tea. The study described in this chapter was designed to determine the effect of tea polyphenols viz. green tea polyphenols (GTP), black tea polyphenols (as water extract of black tea leaves BTE), and their major constituents epigallocatechin-3-gallate (EGCG) and theaflavins (TF), respectively, on the development of prostate tumors differing in androgen-status. Athymic nude mice implanted with tumor xenografts have been widely used as an established pre-clinical in vivo model to assess the effect of drugs/agents on the development of a variety of cancer types (Gupta, 2004 and the references therein).

CWR22Rv1 is an androgen-responsive human CaP cell line derived from a primary tumor that expresses androgen receptors (AR) and secretes prostate
specific antigen (PSA). This cell line is derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski et al., 1999). In nude mice, CWR22Rv1 forms tumors with morphology similar to that of the xenografts, and like the parental CWR22 and CWR22R xenografts, this cell line expresses PSA (Wainstein et al., 1994). On the other hand, PC-3 cells are androgen unresponsive metastatic prostate carcinoma cells, which do not express androgen receptor and are not known to secrete PSA. These two cell lines offer an opportunity to study the differences between the early-to-late CaP development.

Since it is difficult to obtain long-term cultures or cell lines of prostate carcinoma from primary tumors, the use of nude mice as hosts of hetero-transplantation has provided a new dimension to research in cancer including CaP (Gupta, 2004; van Weerden et al., 2000). As a consequence of the absence of functional thymus, the nude mouse has deficient cell-mediated immune response (Stearns et al., 1998). In these mice, humoral antibody formation is only slightly impaired, and the activity of natural killer (NK) cells is actually increased (Stearns et al., 1998). Human xenografts are created by the introduction of human tissues or cell preparations into these immunodeficient rodents. Subcutaneous grafting of human malignant tissues readily results in measurable tumor development in these mice.

Studies described in this chapter have shown that both GTP and BTE as well as their major constituents EGCG and TF, respectively, impart significant chemopreventive/therapeutic response against the development of CWR22Rv1- and PC-3- cell implanted tumors in nude mice, without any toxic response.

5.2 Materials and Methods

Cell Line and Reagents

The androgen-responsive human prostate carcinoma CWR22Rv1 cells were a kind gift from Dr. Todd Thompson (Comprehensive Cancer Center, University of Wisconsin, Madison, WI). The androgen-unresponsive human
prostate carcinoma PC-3 cells were obtained from ATCC (Manassas, VA). The cells were grown in RPMI 1640 (Mediatech, Herndon, VA) with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 95% air and 5% CO₂ humidified atmosphere. Cells grown as mono layer were harvested by brief incubation with 0.05% trypsin-EDTA solution (Invitrogen, Carlsbad, CA) and used for xenograft implantation in nude mice.

Animals and Diet

Athymic (nu/nu) male nude mice were obtained from NxGen Biosciences (San Diego, CA) and housed in the animal care facility of University of Wisconsin, School of Medicine at standard conditions (in laminar airflow cabinets under pathogen-free conditions with 12 h light/12 h dark schedule) and fed with autoclaved Harlan Teklad Sterilizable rodent diet (Madison, WI) ad libitum. A purified preparation of epigallocatechin-3-gallate (EGCG) and theaflavins (TF) (>98% pure) were kind gift from Dr. Yukihiko Hara of Mitsui Norin Co. Ltd. (Shizuoka, Japan). 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%). Black tea leaves were obtained from local market and the water extract was prepared by boiling the tea leaves in distilled water.

Experimental Design

Experimental Design-1: The experimental design of first study was aimed at conducting studies to determine the effect of EGCG against the development of prostate tumor growth in athymic nude mice implanted with androgen-responsive CWR22Rv1 and androgen-unresponsive PC-3 cells. For this purpose, the cells (1 X 10⁶ cells) were suspended in 0.05 ml of medium and mixed with 0.05 ml of Matrigel (basement membrane matrix; Collaborative Biomedical Products, Bedford, MA), and were subcutaneously inoculated on the left and right flanks of each mice. The study protocol was as follows:
Group I: Control; tumors were implanted, no further treatment was given,

Group II: Pre EGCG; a daily treatment of EGCG (1mg/day/mice in PBS, pH 7.4) as intra-peritoneal injections for 7 days followed by implantation of tumors,

Group III: Post EGCG; tumors were implanted first followed by daily intra-peritoneal injections of EGCG (1mg/day/mice in PBS, pH 7.4) for 8 weeks.

Group IV: Pre + Post EGCG; a daily treatment of EGCG (1mg/day/mice in PBS, pH 7.4) as intra-peritoneal injections for 7 days followed by implantation of tumors, the EGCG treatment was continued for 8 weeks.

Throughout the experiment the animals were housed under standard animal housing conditions and had free access to autoclaved water and autoclaved laboratory chow. Blood was withdrawn periodically to determine the effects of treatment on PSA levels in serum.

Experimental Design-2: Based on the outcome of our pilot experiment, we designed a detailed experiment to study and compare the CaP chemopreventive potential of GTP and BTP as well as their major constituents, EGCG and TF, respectively. In this experiment, we employed only the CWR22Rv1 cells for implantation in athymic nude mice. The rationale for this choice is based on the fact that our major goal was to determine the chemopreventive effects of tea polyphenols in early phases of CaP development, when the disease is androgen-dependent. Another reason for excluding PC-3 cell line was that they do not make PSA whereas CWR22Rv1 cells make PSA. In addition, this approach simplified the protocol to make it more manageable, since we were studying a variety of test agents.

For this experiment, the nude mice were randomly divided into five groups of 10 animals each. As described above, CWR22Rv1 cells (1 X 10^6 cells) were suspended in 0.05 ml of medium and mixed with 0.05 ml of Matrigel and were subcutaneously inoculated on the left and right flanks of each mouse. The treatment of mice with tea polyphenols was started 24 hours following the
inoculation of cells. The study protocol of our experimental design was as follows:

Group I: Control; autoclaved water *ad libitum*.

Group II: GTP; freshly prepared solution of 0.1% GTP in autoclaved water (supplied every Monday, Wednesday and Friday) *ad libitum*.

Group III: BTE; freshly prepared 1.25% black tea extract in autoclaved water (supplied every Monday, Wednesday and Friday) *ad libitum*.

Group IV: TF; TF (1 mg/day/mice in PBS; from a stock solution of 50 mM, pH 7.4) given as intra-peritoneal injections.

Group V: EGCG; EGCG (1mg/day/mice in PBS; from a stock solution of 50 mM, pH 7.4) given as intra-peritoneal injections.

In this protocol, to assess the effect of treatments on toxicity, the food consumption and animal body weight was also monitored twice weekly and the fluid consumption was monitored every Monday, Wednesday and Friday throughout the study. In addition blood was periodically withdrawn to determine the effects of treatments on PSA levels in serum.

**Tumor size and volume**

After the development of a measurable tumor, their size was measured twice weekly by determining two perpendicular dimensions and the height using Vernier caliper. The tumor volume was calculated using the formula $V = \frac{1}{2} \left( \frac{4\pi}{3} \right) \left( \frac{L_1}{2} \right) \left( \frac{L_2}{2} \right) (H) = 0.5238 \times L_1 \times L_2 \times H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ is the height. This formula is derived from a formula for calculating the volume of a hemiellipsoid, the geometrical figure most nearly approximating the shape of tumors (Tomayko & Reynolds, 1989). At the termination of these studies, tumors were excised, snap frozen in liquid nitrogen and kept at -80°C for further use.
Collection of blood and determination of serum prostate specific antigen (PSA) levels

The effect of tea polyphenols on CaP development was also determined by following PSA levels in the serum. Blood was collected either by the 'Retro-orbital bleed' or by 'Madibular bleed' and serum separated by allowing the blood to clot at room temperature and centrifuging for 10 min at 4°C.

The levels of PSA were determined commercially (in Experimental Design-1) or by using a quantitative Human PSA enzyme linked immunosorbent assay (ELISA) kit (Anogen, Ontario, Canada) (in Experimental Design-2). This kit utilizes a sandwich enzyme immunoassay technique. In this kit, the microtiter plate is pre-coated with monoclonal antibody specific for PSA. Samples when added to the plates bind to the antibody pre-coated in the wells. A standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for PSA is added to each well to 'sandwich' the PSA immobilized on the plate. The microtiter plate is then incubated for 20-30 minutes followed by thorough washing of the wells to remove all unbound components. Next, a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain PSA and enzyme-conjugated antibody exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at the wavelength of 450±2nm. The intensity of the color measured is proportional to the amount of PSA bound in the initial step. The sample values are then determined using the standard curve.

For PSA determination, serum was used within 24-48 hours of isolation or serum samples were stored at -80°C till further use. PSA standards provided in the kit were reconstituted by dissolving them in appropriate amount of de-ionized water and serum samples were also diluted with de-ionized water if required. 50μl of standards or serum samples were added in the wells of the antibody pre-coated plate followed by 50μl of sample diluent and mixed thoroughly. The plate was incubated at 37°C for 30 minutes followed by a manual washing using a multi channel pipette for a total of five washes followed
by blot drying the plate on absorbent papers. 100μl of conjugate (Anti-human PSA polyclonal antibody conjugated to HRP with preservatives, supplied in the kit) was added to each well and incubated for 30 minutes at 37°C followed by washing the wells as before. 100μl of substrate solution (equal mixture of substrate solution of H₂O₂ and TMB, supplied in the kit) was added and incubated at 37°C for 15 minutes covered with aluminum foil and 100μl of stop solution (2N sulphuric acid solution) was added and mixed well. The plate was read at 450 nm within 30 minutes. A standard curve was made as per the manufacturer’s protocol and the levels of PSA in serum samples were then calculated off the standard curve.

Immunoblot Analysis

For immunoblot analysis, a portion of tumor tissue was thawed on ice and homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 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 to prepare cell lysates. The protein concentration was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL) as mentioned in Chapter III and Western blot analyses were performed. The antibodies used for this purpose were anti-Bax, anti-Bcl-2, anti-PARP (Upsate USA, Charlottesville, VA), anti-VEGF, (Santa Cruz, Biotechnology, Santa Cruz, CA), anti-Caspase 3 (Cell Signaling Technology, Beverly, MA) and anti-β-Actin (Sigma, St Louis, MO).

Following immunoblot analyses, the blots 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.

Statistical Analysis

Results were analyzed using a two-tailed Student’s t-test to assess statistical significance. Values of p<0.05 were considered statistically significant.
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5.3 Results

The major aim of this study was to determine the chemopreventive/anti-proliferative effects of GTP and BTP as well as their major constituents EGCG and TF, respectively, against the development of CWR22Rv1- and PC-3- cell implanted tumors in nude mice.

We first conducted a preliminary study to ascertain the chemopreventive effects of EGCG against the growth and development of CWR22Rv1- and PC-3- implanted tumors in athymic nude mice.

In this study, we assessed the effect of *intra peritoneal* administration of EGCG (1.0 mg/mouse) on CWR22Rv1- and PC-3 human tumor cell growth in nude mice, and ii) serum PSA levels in athymic nude mice bearing CWR22Rv1-tumors. Our interesting data demonstrated that pre- and post- treatments of athymic nude mice, implanted with both androgen-sensitive CWR22Rv1- and androgen-insensitive PC-3- tumors, with EGCG results in a significant inhibition of tumor growth and development (Figures 1-3). However, the inhibition of tumor growth was most dramatic when EGCG was given to mice both before tumor cell implantation and was continued throughout the experimental protocol.

Since serum PSA is arguably considered as a 'gold standard' for monitoring the CaP in human and because the CWR22Rv1 cells make PSA when implanted in nude mice, we studied the effect of EGCG on secreted levels of serum PSA in athymic nude mice implanted with these cells. Our data demonstrated that EGCG treatment resulted in a significant inhibition in serum PSA levels (Figure 4). Interestingly, the effects of EGCG on serum PSA levels were found to exactly correlate the data obtained on tumor growth/development (Figure 1-3). Therefore, the inhibition of PSA was more prominent when EGCG was given to the mice both before tumor cell implantation and was continued throughout the experimental protocol (Figure 4). Tumor growth inhibition data correlated with inhibition in PSA secretion.
In the next step of our approach, we conducted a detailed experiment to study and compare the CaP chemopreventive potential of GTP, BTE and their major constituents, EGCG and TF, respectively. In this experiment, we employed only the CWR22Rv1 cells for implantation in athymic nude mice. In this protocol, to assess the effect of treatments on toxicity, the food consumption and animal body weight was monitored twice weekly and the fluid consumption was monitored every Monday, Wednesday and Friday throughout the study. Our data demonstrated that GTP and BTE given as sole source of drinking fluid did not result in any apparent toxicity in terms of food and fluid consumption as well as body weight (Figures 5 & 6). Similarly, the major ingredients of black and green teas, TF and EGCG respectively, when given as intra-peritoneal injections, did not cause any adverse effect as observed by fluid consumption and body weight (Figures 5 & 6).

Further, treatment of mice with tea polyphenols also did not affect the food intake of the animals (data not shown). These data indicated that treatment of mice with tea polyphenols (oral as well as intra-peritoneal) did not cause any apparent toxicity at the dose levels of the agents tested.

In this study the chemopreventive effects of GTP and BTE (given orally as sole source of drinking fluid) and TF and EGCG (given as intra-peritoneal injections) was studied on tumor growth and development in athymic nude mice implanted with CWR22Rv1 tumors. As shown in Figure 7, the treatment of mice with GTP, BTE and their major constituents EGCG and TF; respectively, was found to result in a significant inhibition in growth of implanted tumors. In our protocol, we terminated the experiment when the tumor reached to a volume of 1200 mm$^3$. Thus, as shown in figure 7, in control animals, the tumor volume of 1200 mm$^3$ was reached in approximately 26 days post-tumor cell inoculation. The most effective tumor growth inhibitory response was observed in GTP-treated group where the targeted tumor volume of 1200 mm$^3$ was reached on day 54 post-tumor cell inoculation. Other treatments were also found to be significantly effective. The tumor volume of 1200 mm$^3$ in animals in BTE group reached in 42 days, whereas it took 43 days and 38 days; respectively, in the animals treated with TF and EGCG.
Figure 1: Effect of EGCG on growth and development of CWR22Rv1- and PC3- cell implanted tumors in athymic nude mice. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1mg/0.1 ml PBS per mouse i.p.), as detailed in 'Materials and Methods'. The control animals received 0.1 ml PBS as vehicle. The photographs were taken on the 30th day following cell implantation.
Figure 2: Effect of EGCG on tumor volume in nude mice implanted with CWR22Rv1- and PC-3- prostate carcinoma cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1mg/0.1 ml PBS per mouse; i.p.), as described in 'Materials and Methods'. The control animals received 0.1 ml PBS as vehicle. The tumor volume was recorded at indicated times. Each value represents the mean ± SE of 4 to 8 tumors (tumor number differs because at some tumor cell-implanted sites there was complete inhibition) in each group.
Figure 3: Effect of EGCG on tumor weight in nude mice implanted with CWR22Rv1- and PC-3- prostate carcinoma cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1mg/0.1 ml PBS per mouse; i.p.), as described in 'Materials and Methods'. The control animals received 0.1 ml PBS as vehicle. The tumor weights were recorded at the termination of experiments times. Each value represents the mean ± SE of 4 to 8 tumors (tumor number differs because at some tumor cell-implanted sites there was complete inhibition) in each group.
Figure 4: Effect of EGCG on serum PSA levels during inhibition of tumor growth and development in athymic nude mice implanted with CWR22Rv1 prostate carcinoma cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with EGCG (1 mg/0.1 ml PBS per mouse; i.p.), as described in 'Materials and Methods'. The control animals received 0.1 ml PBS as vehicle. For determination of serum PSA, at indicated times, the animals were anesthetized and blood was drawn from the peri-orbital venous sinus using heparinized tube. Serum was isolated and PSA analysis was performed by commercial laboratories. Each value represents the mean ± SE from four individual animals.
Figure 5: Effect of tea polyphenols on fluid intake in nude mice implanted with prostate carcinoma CWR22Rv1 cells. In this experiment, the fluid intake was monitored every Monday, Wednesday and Friday and the average fluid intake is plotted as a function of time.
Figure 6: Effect of tea polyphenols on body weight of nude mice implanted with prostate carcinoma CWR22Rv1 cells. In this experiment, body weight was recorded twice weekly throughout the experiment. Body weight of mice is represented as a mean in each group as a function of time.
Figure 7: Effect of tea polyphenols on tumor volume in nude mice implanted with CWR22Rv1 prostate cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols as described in 'Materials and Methods'. The tumor size was recorded at indicated times. Tumor volume was calculated using the formula $V = \frac{1}{2} L_1 L_2 H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ the height.
Days posttreatment

The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols as described in 'Materials and Methods'. The tumor size was recorded at indicated times. Tumor volume was calculated using the formula $0.5 \times L_1 \times L_2 \times H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ the height. Data were computed with Kaplan-Meier analysis using S-plus Software (Insightful; Seattle, WA).

Figure 8: Effect of tea polyphenols on tumor volume in nude mice implanted with CWR22Rv1 prostate cells: A Kaplan-Meier analysis. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols as described in 'Materials and Methods'. The tumor size was recorded at indicated times. Tumor volume was calculated using the formula $0.5 \times L_1 \times L_2 \times H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ the height. Data were computed with Kaplan-Meier analysis using S-plus Software (Insightful; Seattle, WA).
Figure 1: Effect of tea polyphenols on tumor volume in nude mice implanted with CWR22Rv1 prostate cells: A Regression analysis. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols as described in 'Materials and Methods'. The tumor size was recorded at indicated times. Tumor volume was calculated using the formula $0.5238 L_1 L_2 H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ the height. Data were computed as Regression Analysis of Kaplan-Meier curve using S-plus Software (Insightful; Seattle,
Figure 10: Effect of tea polyphenols on tumor volume in nude mice implanted with CWR22Rv1 prostate cells: Log-Rank analysis. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols as described in 'Materials and Methods'. The tumor size was recorded at indicated times. Tumor volume was calculated using the formula $0.5238 \times L_1 \times L_2 \times H$, where $L_1$ is the long diameter, $L_2$ is short diameter and $H$ the height. Data were computed as Log-Rank Analysis of Kaplan-Meier curve using S-plus Software (Insightful; Seattle, WA).
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The data on tumor growth was analyzed using Kaplan-Meier curve, its Regression Analysis as well as by Log-Rank analysis from Kaplan-Meier data. The Kaplan-Meier curve is a nonparametric estimator of distribution function of a time to event variable in the presence of independent censoring (Satagopan et al., 2004). The estimator is also referred to as the Product-Limit estimator because it was first derived as the limiting case of the classical life table estimator of Böhmer in the actuarial science literature for grouped data. The goal of regression analysis is to determine the values of parameters for a function that cause the function to best fit a set of data observations that is provided. The log-rank test is a standard nonparametric test for comparing samples of possibly censored survival times.

Next, we determined the effect of various tea polyphenols on the levels of serum PSA in athymic nude mice implanted with CWR22Rv1 tumors, employing commercially available ELISA kit. A standard curve was made using lyophilized human PSA (supplied with the kit) in the assay to infer the serum levels of PSA (Figure 11).

Our data demonstrated that in all the analyses, GTP treatment was most effective among the agents tested (Figures 8-10). However, BTE, TF and EGCG treatments were found to impart significant tumor growth inhibitory effects (Figures 8-10). As shown in figure 12, at 3 weeks post-tumor inoculation, the level of PSA in control animals was found to be 13.95±0.82 ng/ml; whereas in animals treated with GTP, BTE, EGCG and TF, the PSA levels were found to decrease to 4.95±1.23, 6.37±1.75, 4.07±0.98 and 5.9±0.78 ng/ml. Similarly at 4 weeks post inoculation, the level of PSA in control animals was 26.4±1.32 ng/ml; whereas in animals treated with GTP, BTE, EGCG and TF, the PSA levels were found to decrease to 4.46±1.32, 8.85±2.32, 5.7±0.58 and 6.9±1.32 ng/ml (Figure 12). PSA levels were also determined at earlier times (data not shown); however, the values were found to be less than 4 ng/ml (considered negative as per the criterion of the kit), the data is not shown here. Thus, our data clearly demonstrated that GTP and BTE as well as their major constituents resulted in a significant decrease in the serum PSA levels in athymic nude mice implanted with human CaP cells.
Figure 11: Standard Curve for PSA measurement. The PSA standard samples obtained with the kit were reconstituted with de-ionized water and run along with samples to obtain the standard curve, as per the manufacturer's protocol. Absorbance of the developed color in the reaction was measured spectrophotometrically at the wavelength of 450±2 nm.
Figure 12: Effect of tea polyphenols on serum PSA levels during inhibition of tumor growth and development in athymic nude mice implanted with CWR22Rv1 prostate carcinoma cells. The cells (in matrigel) were implanted on both flanks of nude mice. The animals were treated with tea polyphenols, blood was obtained, serum was isolated and PSA levels were measured as described in 'Materials and Methods'.

** indicates p<0.05
Using this standard curve, the effect of tea polyphenols on PSA levels were determined. As shown by the data in figure 12, tea polyphenols resulted in significant inhibition of PSA at 3 and 4 weeks post implantation of CWR22Rv1 cells.

Our next aim was to determine the mechanisms of prostate tumor growth inhibition in nude mice. Because tea polyphenols have been shown to induce apoptosis of cancer cells, we determined the effect of tea polyphenols on PARP, Caspase 3 and Bcl family of proteins, all of which are known to regulate programmed cell death (Adhami et al., 2003; Shukla & Gupta, 2004; Cosulich et al., 1999). As shown by the western blot analysis, the tea polyphenols were found to increase in active caspase-3 and cleaved PARP levels indicating an induction of apoptosis by tea polyphenols. Interestingly, tea polyphenols resulted in an appreciable decrease in the levels of pro-form of caspase-3. We next studied the effect of tea polyphenols on Bax and Bcl-2 protein, which are the most important members of Bcl-2 family of proteins. As shown in figure 14, tea polyphenols resulted in an appreciable upregulation in Bax and decrease in Bcl-2 proteins. These results suggested that the tumor inhibitory effects of tea polyphenols are mediated via an increased apoptosis in tumors and this induction of apoptosis is caused via a modulation of Bcl-2 family proteins that favors apoptosis.

Because our studies described in chapter IV have shown that GTP causes an inhibition of angiogenesis in prostate, we next compared the effects of various tea polyphenols on VEGF protein levels in prostate tumors in nude mice. As expected, the expression in VEGF was inhibited by all the preparations of tea polyphenols examined (Figure 15).
Figure 13: Effect of tea and its polyphenols on the protein levels of Caspase 3 and cleaved-PARP during inhibition of CWR22Rv1 prostate carcinoma tumors in athymic nude mice. At the termination of experiment, the tumors were surgically removed, protein lysates were prepared and Western blot analyses were performed using appropriate antibodies as detailed under ‘Materials and Methods’. Blots were stripped and reprobed for β-actin to confirm the equal loading of the proteins.
Figure 14: Effect of tea and its polyphenols on the protein levels of Bcl-2 and Bax during inhibition of CWR22Rv1 prostate carcinoma tumors in athymic nude mice. At the termination of experiment, the tumors were surgically removed, protein lysates were prepared and Western blot analyses were performed using appropriate antibodies as detailed under 'Materials and Methods'. Blots were stripped and reprobed for β-actin to confirm the equal loading of the proteins.
Figure 15: Effect of tea and its polyphenols on the protein levels of VEGF during inhibition of CWR22Rv1 prostate carcinoma tumors in athymic nude mice. At the termination of experiment, the tumors were surgically removed, protein lysates were prepared and Western blot analyses were performed using appropriate antibodies as detailed under ‘Materials and Methods’. Blots were stripped and reprobed for β-actin to confirm the equal loading of the proteins.
Studies in this chapter were designed to compare the cancer chemopreventive/therapeutic effects of a variety of tea polyphenols against CaP in athymic nude mice model that is regarded to possess relevance to human CaP. We have also studied the involvement of apoptosis and Bcl-2 family protein as a mechanism of tumor inhibition by tea polyphenols.

It has been shown that active constituents of tea can offer protection against all stages of carcinogenesis (tumor initiation, promotion and progression), because of its antioxidant properties against free radicals, blocking signal transduction and nuclear oncogene expression, trapping of ultimate carcinogens and inducing apoptosis and cell cycle arrest (Lambert & Yang, 2003; Yang et al., 2002; Yang et al., 2000; Yu et al., 2004; Kelly et al., 2001; Baatout et al., 2004). Recent studies have indicated that multiple mechanisms may be involved in CaP chemopreventive effects of tea polyphenols.

To study the comparative chemopreventive/therapeutics potential of different preparations of tea polyphenols, we designed our study with a two-phase approach. In the first phase, we conducted a pilot study to determine whether or not a detailed study should be undertaken to assess the chemopreventive effects of tea polyphenols. In this pilot study, we assessed the effect of intra-peritoneal administration of EGCG (a well studied and considered to be the major active ingredient of green tea) on prostate tumor growth in nude mice implanted with androgen-dependent CWR22Rv1 as well as androgen-independent PC-3 cells. Our data demonstrated that EGCG significantly inhibits i) the growth of implanted tumors in athymic nude mice, and ii) serum PSA levels. Based on the outcome of this pilot study, we designed the detailed study with GTP and BTE and their major polyphenolic constituents EGCG and TF, respectively. For this detailed study, we selected CWR22Rv1 cell because these cells are known to secrete PSA.

As discussed under ‘Results’ section, our data clearly demonstrated that all the preparation of tea polyphenols cause a significant inhibition in tumor growth and the serum PSA levels in athymic nude mice implanted with
CWR22Rv1 cells, which are known to secrete PSA. This is an important observation because serum PSA is considered to be an important marker for identifying humans for adenocarcinoma of the prostate (Lieberman, 2004; Gonzalez et al., 2004). In the clinic, serum PSA measurements are being widely used to screen for CaP (Linton & Hamdy, 2004; Passadakis et al., 2004; Van Der Cruijsen-Koeter et al., 2001; Özden et al., 2003). Several investigators have reported the usefulness of serum PSA as a follow up marker for local recurrence and/or distant disease in the patients after radical prostatectomy, radiation and hormonal therapy (Gonzalez et al., 2004; Lieberman, 2004). Despite of a continuing debate, at the present time, serum PSA is the regarded as the best marker for volume of CaP (tumor burden) in men (So et al., 2003; Ornstein and Pruthi, 2000; Han et al., 2000).

Our data also demonstrated that GTP, BTE or their major constituents EGCG and TF, at observed doses, did not cause any apparent toxicity (body weight and fluid consumption) in mice. Further, there was no observed change in consumption of food or fluid in various treatment groups. This is consistent with earlier studies where tea and its polyphenols did not show any apparent sign of toxicity in vivo (Adhami et al., 2003a; Cao & Cao, 1999; Gupta et al., 2001 and the references therein; Gupta, 2004; Wang et al., 1994).

Next, we assessed the mechanisms involved in the CaP chemopreventive effects of tea polyphenols. Our data suggested that the growth inhibition of CWR22Rv1 tumor xenograft by tea polyphenols is associated with an increase in apoptosis of tumor cells. The induction of apoptosis was evident from our data showing the increase in active-caspase-3 and cleaved PARP levels in the tumors. Caspase 3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the sequence of events associated with apoptosis. Caspase 3 cleaves a variety of cellular molecules that contain the amino acid theme DEVD such as poly ADP-ribose polymerase (PARP), the 70 kD protein of the U1-ribonucleoprotein and a subunit of the DNA dependent protein kinase. Thus, our findings are consistent with in vitro studies where treatment of CaP cells with tea polyphenols resulted in apoptosis and cell cycle arrest (Ahmad et al., 1997; Agarwal R, 2000; Brusselmans et al., 2003; Chung et al., 2001; Gupta et al., 2000).
Further, our study demonstrated that increased apoptosis in the tumors of is associated with down modulation of Bcl-2 and a concomitant increase in Bax. Bcl-2 is an anti-apoptotic gene and, in fact, the link between apoptosis and cancer, emerged when Bcl-2 (B-cell lymphoma 2), which is the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death (Korsmeyer, 1992). This unexpected discovery gave birth to the concept, now widely embraced that impaired apoptosis is a crucial step in the process of cancer development (Heiser et al., 2004; Kitada et al., 2002 and the references therein). In the present study, we have shown that tea polyphenols resulted in a significant decrease in the levels of anti-apoptotic Bcl-2 protein and increase in the pro-apoptotic Bax protein thus shifting the Bax/Bcl-2 ratio in favor of apoptosis. Studies have shown that Bcl-2 forms a heterodimer with Bax and might thereby neutralize its pro-apoptotic effects (Hirotani et al., 1999; Ottilia et al., 1997). In addition, Bcl-2 is also known to prevent the release of caspases (Brito et al., 2003; Okuno et al., 1996; Kluck et al., 1997). It is also important to mention here that our findings are consistent with other studies where a similar observation in cell culture was observed (Adhami et al., 2003b; Shukla & Gupta, 2004; Cosulich et al., 1999). Thus, our data suggested that the observed tumor inhibition by tea polyphenols may be associated with modulations in Bcl-2-family proteins that favor apoptosis of the tumor cells in vivo.

We also determined the modulations in VEGF, a marker protein for angiogenesis, during the observed tumor inhibition by tea polyphenols. Angiogenesis is a physiological process of formation of new blood vessels and this process is always stimulated in tumors (Ribetti & Vaccà, 1994; Nor et al., 1999). Our data demonstrated that the protein level of VEGF was appreciably inhibited by tea polyphenols. The inhibition of VEGF by green tea has previously been shown in a model of corneal neovascularization (Cao and Cao, 1999). In this model, drinking green tea (1.25% in drinking water) was found to be 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.

Taken together, our study suggested that all the preparation of tea (black tea as well as green tea) were effective in inhibiting the growth of prostate tumors in athymic nude mice. Our data also suggested that the CaP chemopreventive effect of tea polyphenols are mediated via i) induction of apoptosis of tumor cells that is caused by modulations in Bcl-2-family proteins, which favor apoptosis, and ii) inhibition of angiogenesis in tumors. The outcome from this study could have a direct practical implication and translational relevance to human CaP patients.

References


Chapter V


Sartor L, Pezzato E, Dona M, Del'Alca I, Calabrese F, Morini M, Albini A, Garbisa S (2004). Prostate carcinoma and green tea: (-)epigallocatechin-3-


