Prostate Cancer Chemoprevention by Green Tea: In Vitro and in Vivo Inhibition of Testosterone-mediated Induction of Ornithine Decarboxylase

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ABSTRACT

Recently, we have shown that ornithine decarboxylase (ODC), a rate-controlling enzyme in the polyamine biosynthetic pathway, is overexpressed in prostate cancer (PCA) and prostatic fluid in humans (R. R. Mohan et al., Clin. Cancer Res., 5: 143–147, 1999). ODC is also characterized as an androgen-responsive gene, and the androgenic stimulation regulates the development and growth of both normal and tumorigenic prostate cells. Thus, chemopreventive approaches aimed toward the modulation of ODC could be effective against PCA. Green tea polyphenols (GTPs) possess strong chemopreventive properties against a variety of animal tumor models and in some human epidemiological studies. At least two epidemiological studies have suggested that people who consume tea regularly may have a decreased risk of PCA. In this study, we investigated the effect of GTPs against testosterone-mediated induction of ODC in human prostate carcinoma cells, LNCaP as an in vitro model, and in Cpb:WU rats and C57BL/6 mice as in vivo models. Treatment of LNCaP cells with testosterone resulted in induction of ODC activity in a dose-dependent manner. Pretreatment of the cells with GTPs resulted in a significant inhibition of testosterone-induced increase in ODC activity. Similar effects of GTPs were observed in anchorage-independent growth assay of LNCaP cells where pretreatment of the cells with GTP was found to result in dose-dependent inhibition of colony formation. Testosterone treatment of the cells resulted in a significant increase in the level of ODC mRNA, and this increase was almost completely abolished by prior treatment of the cells with GTPs. The administration of testosterone (10 mg/kg body weight, i.p.) to sham-operated and castrated Cpb:WU rats resulted in 2- and 38-fold increases in ODC activity, respectively, in the ventral prostate. Oral feeding of 0.2% GTPs in drinking water for 7 days before testosterone administration resulted in 20 and 54% decreases in testosterone-mediated induction of ODC activity in sham-operated and castrated rats, respectively. Similar results were obtained with C57BL/6 mice, where testosterone treatment at similar dosage resulted in a 2-fold increase in ODC activity in the ventral prostate and prior oral feeding with 0.2% GTPs resulted in 40% inhibition in this induction.

INTRODUCTION

In the United States, PCA is the most common cancer and the second biggest cause of cancer-related deaths in males (1, 2). According to an estimated projection by the American Cancer Society, a total of 184,500 were expected to be diagnosed with PCA in the United States in the year 1998, and 39,200 PCA-related deaths were predicted (2). To fight against PCA, a bidirectional approach aiming toward: (a) defining the mechanism-based and treatment/therapy-responsive markers for the development of PCA; and (b) developing novel chemopreventive strategies may be extremely helpful. Regulation of normal growth, development, and function of the prostate gland is intimately associated with androgen action because many androgen-regulated genes are known to contribute to development/progression of PCA (Refs. 3 and 4 and references therein). Certain important androgen-regulated genes, e.g., prostate-specific antigen and KLK-2 (5), C(3) protein (6), Slp (7), and probasin (8) have been well characterized for their roles in mitogenic signaling pathway in the progression of PCA.

Studies have demonstrated that prostate contains some of the highest concentrations of polyamines and polyamine-metabolizing enzymes (9, 10). ODC is the first and the rate-controlling enzyme in the polyamine biosynthetic pathway and catalyzes the conversion of ornithine to putrescine (11). Our recent studies have shown that ODC activity is significantly higher in PCA tissue than in paired normal tissue and prostatic fluid in patients with PCA (12). This study suggests that ODC could serve as a target for prevention and therapy of human PCA. Consistent with this suggestion is the fact that DFO, a suicide substrate inhibitor of ODC, is presently being evaluated for the prevention and/or treatment of PCA and other cancer types in humans (Refs. 13–15 and references therein). ODC is believed to possess an important role in the regulation of cell growth, proliferation, and differentiation (Refs. 11–15 and references therein). On the basis of differential display technique, Liang and Pardee (16) have identified ODC as an androgen-responsive gene, and it is now well established that the androgenic stimulation regulates the development and growth of both normal as well as tumorigenic prostate cells. Huggins and Hogg (17) have demonstrated that androgen ablation therapy exerts beneficial effects in the patients with advanced PCA. In recent years, it is increasingly realized that the chemopreventive approaches aimed toward the modulation of ODC may be effective against PCA (18, 19).

Studies have shown that green tea, a popular beverage consumed worldwide, affords cancer chemopreventive effects in a variety of animal tumor model systems (Refs. 20–24 and references therein). Most of these studies have been conducted with a polyphenolic mixture obtained from green tea (GTP). GTP has also been demonstrated to inhibit ODC induction caused by tumor promoters in mouse skin and other tissues (25–28). Japanese and Chinese populations, which regularly consume green tea, have the lowest prostate cancer incidence (29, 30). Two epidemiological studies indicate that people who consume tea regularly may have a decreased risk of PCA (31, 32). In addition, in one study the major constituent of green tea EGCG has been shown to cause growth inhibition and regression of human prostate and breast tumors in athymic nude mice (33). Our working hypothesis is that testosterone-mediated induction of ODC is an important contributor to PCA development, and GTP by inhibiting ODC induction will result in prevention of PCA. Validation of this hypothesis could lead to novel strategies for developing preventive approaches for human PCA.

This study is designed to investigate both under in vitro and in vivo situations: (a) the effect of testosterone on cell growth/proliferation and ODC activity; and (b) whether GTP can ameliorate these responses.

MATERIALS AND METHODS

Materials. A polyphenolic fraction was prepared from green tea (hereafter referred to as GTPs) as per method standardized in our laboratory (26). Briefly, dried green leaves (100 g) were extracted twice with hot water (80°C) and three times with 80% ethanol (700 ml each) and then dried in a convection oven. The combined extract (3.5 liters) was concentrated under vacuum to 1 liter and then extracted with an equal volume of chloroform. The aqueous layer was extracted three times with ethyl acetate (800 ml each time) under nitrogen, and
the total organic soluble fraction (2.4 liters) was concentrated under vacuum. The residue obtained was dissolved in water (50 ml) and freeze-dried. The light brown solid matter obtained was called GTPs. Chromatographic analysis of this mixture showed that it contains four major polyphenolic compounds, i.e., EGCG, epicatechin-3-gallate, epigallocatechin, and epicatechin, which contain ~36% (by weight) of fresh green tea leaves (26). We have shown earlier that this method yields GTP that approximately contains 61, 27, 5, and 6% (percent of total epicatechin derivatives, w/w) of EGCG, epicatechin-3-gallate, epigallocatechin, and epicatechin, respectively (26).

**Cells and Treatment.** Human prostate carcinoma cells, LNCaP, were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were cultured in a humidified atmosphere of 95% air/5% CO2 in an incubator.

To study the effect of testosterone on the ODC activity, the cells (80% confluence) were treated either with ethanol (final concentration, 0.1%) or specified dose of testosterone in the same volume of ethanol. At 10 h post-treatment, the medium was removed; cells were washed twice with ice-cold PBS, and harvested cells were processed for further study. To study the effect of GTP on testosterone-caused induction of ODC activity, the cells, (80% confluent) were pretreated with either sterile water or varying doses of GTP (10–80 μM) in sterile water. After 1 h, the cells were treated with ethanol alone (control) or testosterone (50 nM in ethanol). Two h later, the cells were harvested, washed with PBS, and processed as desired.

**Animals and Treatment.** The castrated and sham-operated Cpb:WU rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were acclimatized for 1 week. Animals were fed Purina chow and water ad libitum. The castrated and sham-operated rats were divided into four groups (containing four animals in each group). Rats in groups I and II were fed with normal drinking water, whereas the animals in groups III and IV were fed with 0.2% GTP (w/v) in drinking water as a sole source of drinking water. The feeding regimen was followed for 7 days, and on the eighth day, rats in groups II and IV received s.c. injections of testosterone (10 mg/kg body weight in corn oil; testosterone was dissolved in 100% ethanol and was diluted with corn oil). Animals of groups I and III were injected only with corn oil as vehicle. The feeding regimen of GTP used here was based on our experience and published work from other laboratories (Refs. 25 and 26 and references therein). Twenty-four h after testosterone administration, all the animals were sacrificed, and ventral prostate tissue from each animal was collected and immediately processed for ODC activity determination.

In addition, the male C57BL/6 mice (17–18 weeks of age; obtained from Harlan Sprague Dawley, Inc.) were acclimatized for 1 week and fed ad libitum with Purina chow diet and water. To assess the effect of GTP on testosterone-caused induction of ODC, the animals were divided into four groups consisting of six mice each. A similar treatment protocol as described for rats above was followed.

**ODC Enzyme Activity Assay.** The cytosolic fraction was prepared from the cells and the tissues by the procedure standardized in our laboratory (12, 26). The ODC activity was determined in the cytosolic fractions thus obtained by measuring the release of 14CO2 from the [14C] ornithine as described earlier (12, 26). Briefly, the assay mixture contained 35 mM sodium phosphate buffer (pH 7.2), 0.2 mM pyridoxal phosphate, 4 mM DTT, 1 mM EDTA, and 0.4 mM L-ornithine containing 0.5 μCi of [1-14C] L-ornithine hydrochloride. After incubation at 37°C for 1 h in 15-ml Corex centrifuge tubes equipped with rubber stoppers and center well assemblies, the reaction was stopped by adding 0.5 ml of 2 M citric acid. The incubation was continued for another 1 h to ensure complete absorption of 14CO2 by the ethanolamine and methoxyethanol (0.2 ml; 2:1, v/v) contained in the center well. Finally, the center well containing the ethanolamine/methoxyethanol mixture was transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml of ethanol. The radioactivity was measured in a Beckman LS 6000 SC liquid scintillation counter with 95% efficiency. Enzyme activity is expressed as pmol 14CO2 released/h/mg protein. Assays were carried out in triplicate; blank assays contained no enzyme.

**ODC mRNA Expression.** The LNCaP cells cultures grown to 80% confluence were pretreated with either sterile water or 40 μg of GTP/ml medium, and 1 h later, they were treated with alcohol alone or 50 nM testosterone dissolved in alcohol. This dose of GTP was selected because it produced an inhibitory effect on ODC activity. The cells were harvested 2 h after the last treatment, and total RNA was extracted by the guanidine thiocyanate method as described earlier (34). Equal amounts of RNA (25 μg/lane) were fractioned in the presence of ethidium bromide by denaturing agarose gel electrophoresis and transferred to a Zeta Probe membrane. RNA was quantified in water by a spectrophotometric assay at 260-nm wavelength. The Zeta Probe membrane was hybridized with 32P-labeled ODC antisense probe (>2.1 kilobase) and washed as described previously (34). Rat GAPDH cDNA (~1.3 kb) labeled with 32P by random primer was used for normalization of mRNA for ODC. Autoradiographs were obtained by exposing the membrane for 12–24 h at ~70°C.

**Soft Agar Colony Formation Assay.** LNCaP cells were cultured under the conditions detailed above. For anchorage-independent cell growth, a soft agar colony formation assay was performed in a six-well plate. Each well contained 2 ml of 0.5% agar in medium as the bottom layer, 1 ml of 0.38% agar in medium, and 1000 cells at the feeder layer. The cells were pretreated with various concentrations of GTP (10–50 μg in medium) for 12 h, followed by treatment with testosterone (50 nM with 1 ml of 0.38% agar in medium with ethanol) as the top layer. Three wells were used for each concentration. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100, and a group of >20 cells were counted as a colony.

**RESULTS**

**Induction of ODC by Testosterone in LNCaP Cells.** We first determined the effect of testosterone on ODC activity in LNCaP cells. As shown by data in Fig. 1A, the treatment of cells with testosterone resulted in a dose-dependent induction of ODC activity, with maximum effects achieved at a 50 nM dose and further increase showed a decreasing trend. Time-dependent studies on the kinetics of ODC...
induction by a 50 nM dose of testosterone resulted in maximum induction of ODC at 2 h posttreatment (Fig. 1B). Therefore, to evaluate the effect of GTP on testosterone-induced ODC activity, we selected the 50 nM dose and 2 h of testosterone treatment.

**Inhibition of Testosterone-mediated Induction of ODC Enzyme Activity and ODC mRNA by GTP in LNCaP Cells.** As shown by data in Fig. 2, the pretreatment of LNCaP cells with varying concentrations of GTP resulted in significant inhibition of testosterone-caused induction of ODC activity in a dose-dependent fashion. In additional studies, we performed Northern blot analysis to investigate the effect of GTP on testosterone-mediated modulation in ODC mRNA expression. As shown in Fig. 3, the 32P-labeled ODC cDNA probe hybridized to a single mRNA species of ∼2.0 kb. The data demonstrate that compared with vehicle-treated control (Lane 1), treatment of cultures with testosterone (50 nM) for 2 h (Lane 2) resulted in a significant increase in ODC mRNA expression. However, the treatment of cells with GTP (40 μg/ml) 1-h prior to that of testosterone resulted in almost complete inhibition of testosterone-caused induction of ODC mRNA expression.

**Inhibition of Testosterone-mediated Colony Formation of LNCaP Cells by GTP.** In the next series of experiments, we investigated the effect of GTP with and without testosterone on soft agar colony formation. As shown in Fig. 4, compared with the vehicle-treated control (60 colonies/well), the treatment of LNCaP cells with testosterone resulted in 288 colonies/well, which accounts for a 5-fold increase in the colony formation. A significant increase in the size of colony was also observed in testosterone-treated cells (Fig. 4A). The pretreatment of the cells with GTP resulted in a dose-dependent inhibition of colony number and size in these cells. The pretreatment with GTP at 10, 20, 40, 60, and 80 μg/ml resulted in 240, 212, 172, 121, and 83 colonies/well, respectively (Fig. 4B). Thus, as compared with the testosterone-treated cells (288 colonies/well), GTP pretreatment resulted in a 17, 26, 40, 58, and 71% inhibition of the colony formation at 10, 20, 40, 60, and 80 μg/ml doses, respectively.

**Inhibition of Testosterone-mediated Increase in ODC Enzyme Activity in Cpb:WU Rats by GTP.** To investigate the relevance of our in vitro findings under in vivo situation, we used castrated and sham-operated Cpb:WU rats. As shown in Fig. 5, the administration of testosterone to both castrated and sham-operated rats resulted in a significant increase in ODC activity in the ventral prostate. The selection of the ventral prostate region in the castrated rats for these studies was based on the fact that compared with other prostate regions, a significantly higher ODC activity was shown to be associated with ventral prostate, followed by dorsal prostate in intact adult rats (35). However, with castrated rats, ODC activity was reduced to almost negligible units in all prostate regions (35, 36). Administration of testosterone resulted in 2- and 38-fold increase in ODC activity in sham-operated and castrated rats in the ventral prostate (Fig. 5). The oral feeding of GTP in drinking water for 7 days at the dose of 0.2% (w/v) as a sole source of drinking water resulted in 20 and 54% decrease in testosterone-caused induction of ODC activity in the ventral prostate of sham-operated and castrated rats, respectively (Fig. 5). Feeding of GTP alone, however, did not result in any noticeable alteration in ODC activity in both sham-operated and castrated rats.

**Inhibition of Testosterone-mediated Increase in ODC Enzyme Activity in C57BL/6 Mice by GTP.** To further confirm the putative androgen-regulated activity of ODC in the prostate, we also conducted experiments in C57BL/6. Data in Fig. 6 demonstrate that the administration of testosterone resulted in 2-fold increase in the activity of ODC in the ventral prostate when compared with the control group fed on normal water. The animals fed GTP experienced a 40% inhibition in testosterone-caused induction of ODC activity in the ventral prostate (Fig. 6). Feeding of GTP alone, however, did not result in any significant alteration in the ventral prostate ODC activity.

**DISCUSSION**

The limited treatment options and diagnostic approaches as well as poor treatment success rate make PCA one of the leading causes of death, accounting for 13% of the total cancer-related deaths in American males (1, 2). In this scenario, mechanism-based approaches are needed to develop novel strategies against PCA. Chemoprevention by naturally occurring compounds appears to be a practical approach to fight with PCA. In our recent studies, we provided evidence that ODC activity is overexpressed in prostate tissue and prostatic fluid in naturally occurring compounds.
humans with PCA (12). These data re-enforce the suggestion that ODC could be an important marker for PCA and may also serve as a target to develop novel chemopreventive strategies against PCA.

ODC is the first and the rate-limiting enzyme in the polyamine biosynthetic pathway in the mammalian cells (Ref. 11 and references therein). Studies have provided convincing evidence for a role of polyamines and ODC in tumor cell growth and in the biological response of tumor promoters and growth factors (11–13, 19). Much of these studies are conducted in mouse skin (37, 38), where ODC is tightly regulated in normal tissues at the mRNA and protein levels and is dysregulated in papillomas and carcinomas (39). The “basal” level of ODC activity is found to be significantly elevated in mouse skin papillomas when compared with the normal epidermis (40, 41). On the basis of these observations, it is suggested that an uncontrolled regulation of ODC may be an important contributor to tumor development in epithelial tissues. ODC is ubiquitously expressed in most of the tissues in humans and experimental animals (42, 43), and in humans, among all of the organs, the highest concentration of ODC occurs in the prostate (9, 10, 12, 44).

It is also important to emphasize here that the normal growth and proliferation of the prostate is androgen dependent. In the prostate, ODC is regulated by androgens, probably via the androgen receptor binding consensus sequence within its tumor promoter (45). Studies have shown that the androgen replacement therapy in castrates and androgen supplementation in intact rats increases ODC activity (35, 36). Similarly, in AXC rats, levels of ODC message and activity in the ventral prostate is shown to cause an age-dependent decrease that can be overcome with testosterone supplementation (46). All of these studies suggest that ODC could be a target for prevention and therapy of PCA.

Extensive studies from this and other laboratories have verified cancer chemopreventive effects of green tea polyphenols against many animal tumor bioassay systems (Refs. 20–28 and references therein). In these studies, oral consumption or topical application of GTP or its constituents has been shown to offer protection against all stages of multistage carcinogenesis that include initiation, promotion, and progression. Epidemiological studies, although inconclusive, also provided evidence that regular consumption of green tea may be associated with the reduced risk of cancer development in many.
organs (31, 47–49). We have also shown that green tea constituent EGCG induces apoptosis in human prostate carcinoma cells DU145 (50). The present study is designed to investigate our hypothesis that testosterone-mediated induction of ODC may be an important contributor of the development of PCA, and GTP is capable of inhibiting the development of prostate cancer both under in vitro and under in vivo situations by targeting at ODC.

Androgens are essential for normal prostate physiology and have been shown to play key role in the development and pathogenesis of PCA (51, 52). Studies have also shown that androgen deprivation may prevent the development of PCA (17). The importance of androgen in the development of PCA can also be gauged from the fact that in eunuchs the occurrence of PCA is very rare (18, 19, 51, 52). Therefore, we used androgen-sensitive LNCaP cells for our in vitro studies. We examined the effect of testosterone and/or GTP on modulations in ODC. Our data demonstrated a significant increase in the level of ODC enzyme activity as well as ODC mRNA in response to testosterone and a dose-dependent inhibition by GTP. The androgen-mediated up-regulation of ODC mRNA has earlier been shown in a study by Betts et al. (53), which suggested that ODC regulation by androgen is mediated through androgen receptor protein and is independent of secondary protein synthesis. ODC is regarded as a marker for increased cellular growth/proliferation, which if not controlled, could result into the development of cancer. The down-regulation of ODC, in PCA cells, by GTP is an important observation. However, the effect of GTP on the level of ODC in normal prostate cells is not clear at present. Because GTP and/or its constituents are shown to have selective growth-inhibitory response toward cancer cells (20, 50), we believe that the ODC-inhibitory response of GTP might be selective only for cancer cells, with no effect on the normal cells. Further studies are, however, needed to support this speculation. We also studied the effect of testosterone and/or GTP on the anchorage-independent growth of LNCaP cells by soft agar colony-formation assay. Our data showed a significant increase in the ability of the cells to form colonies by testosterone, which was inhibited by GTP in a dose-dependent fashion.

Our next objective was to investigate whether these in vitro findings could also be translated to in vivo situation. For this, we selected Cpb:WU rat as the animal model and studied the effect of testosterone-mediated alterations in ODC activity. This choice was based on the fact that carcinogenesis, in the accessory sex glands of these animals, is dependent both on exposure to the chemical carcinogen and on chronic hormonal stimulation by testosterone (54). Our data demonstrated that androgen (testosterone) plays an important role in regulation of ODC in the ventral prostate. The two important observations of the present study were that: (a) the castration of Cpb:WU rats resulted in almost negligible ODC enzyme activity compared with that in sham-operated animals; and (b) the testosterone-mediated induction of ODC activity was much more pronounced in sham-operated Cpb:WU rat prostate than in castrated rats. These observations further strengthened our hypothesis that androgen-mediated up-regulation of ODC is an important contributor toward the...

Fig. 5. Testosterone-caused induction of ODC enzyme activity in ventral prostate of sham-operated (A) and castrated (B) Cpb:WU rats and its inhibition by oral feeding of GTP. Each column represents the mean of data obtained from four individual rats, and each assay was done in duplicate; bars, SD.

Fig. 6. Testosterone-caused induction of ODC enzyme activity in ventral prostate of C57BL/6 mice and its inhibition by oral feeding of GTP. Each column represents the mean of data obtained from six individual mice, and each assay was done in duplicate; bars, SD.
development of PCA. Furthermore, we also conducted similar studies in the C57BL/6 mouse because this mouse model, among many other inbred, hybrid, and outbred strains, is considered resistant to tumor promotion (55). The data obtained with this animal model were similar to those of Cpb-Wu rats. Consistent with our in vitro results, in these animal models under in vivo situation, GTP-feeding resulted in a significant inhibition of ODC enzyme activity. Taken together, this study has demonstrated that GTP inhibits the androgen-mediated cell growth and ODC in prostate cancer cells. These data imply that ODC can be used as target for the prevention of PCA and that GTP, and its polyphenolic constituents merit further investigation for developing strategy against PCA.

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