ABSTRACT

Although the etiology of prostate cancer is still not clear, family history, hormones, and age are thought to play a role in its initiation and progression. There is no cure for the advanced disease. Because prostate cancer initially develops as an androgen-dependent tumor, agents with antian- drogen activity have become the focus for chemoprevention of this disease. A pilot study was undertaken to test the efficacy of flutamide (an antian- drogen) in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer. Three groups of mice received s.c. implantation of slow-release flutamide pellets: (a) low-dose flutamide group (6.6 mg/kg); (b) high-dose flutamide group (33 mg/kg); and (c) control placebo group. Efficacy was measured by the absence of palpable tumor formation. Prostate tissues/tumors were harvested for evaluation by molecular and histology techniques. The low-dose flutamide group did not differ significantly from the placebo group, in which palpable tumors initially presented at 17 weeks of age, and by 33 weeks, all of the animals developed palpable tumors. In the high-dose flutamide group, however, tumors did not appear until 24 weeks, a lag of 7 weeks, and by 34 weeks, 42% of the animals were still tumor free. The period of time at which 50% of the animals had tumors was 33 weeks in the high-dose flutamide group, 24.5 weeks in the low-dose flutamide group, and 24.5 weeks in the placebo group. The difference between the placebo and high-dose flutamide groups was statistically significant (log rank, \( P = 0.0036 \); Wilcoxon's statistical analysis, \( P = 0.0060 \)). Tumors from high-dose flutamide-treated animals were more differentiated and retained much of the normal glandular architecture compared with those of the placebo group, whose tumors consisted of sheets of poorly differentiated cells. The expression of T antigen in the prostate tissues of flutamide-treated animals (at 10 weeks age) was lower than that in the comparable placebo-treated group. Flutamide had the ability to suppress T antigen-driven carcinogenesis, resulting in a significant decrease in the incidence of prostate cancer and an increase in the latency period of prostate cancer in TRAMP mice.

INTRODUCTION

Prostate cancer is one of the most frequent cancers among men in the United States, with more than 184,500 new cases expected this year (1). Unfortunately, over 60% of these newly diagnosed cases of prostate cancer will be pathologically advanced; at this stage, there is no cure, and the prognosis is dismal. The frequency of latent prostatic tumors has been shown to increase with each decade of life from the 50s (5.3–14%) to the 90s [40–80% (2)]. Thus, one approach may be early detection of prostate cancer through screening programs to reduce the number of patients with advanced prostate cancer. Another strategy is to develop drugs that may prevent prostate cancer.

Hormones, age, and family history are thought to play a role in the initiation and progression of prostate cancer, which initially develops as an androgen-dependent tumor (3, 4). The premalignant lesion then progresses to hormone-independent adenocarcinoma that eventually spreads to the bone. Although androgen ablation at this hormone-refractory stage is ineffective, androgen deprivation strategy as an early intervention may delay the initiation, promotion, and/or progression of prostate cancer, resulting in reduced morbidity and mortality. Approaches to influence tissue androgen levels include: (a) inhibiting the pituitary secretion of luteinizing hormone by luteinizing hormone-releasing hormone analogues; (b) preventing the conversion of testosterone to dihydrotestosterone by 5α-reductase in the prostate; and (c) blocking the prostatic androgen receptors by using steroid-like antagonists with no intrinsic activity to reduce the potentially unacceptable systemic toxicity. One such agent may be the nonsteroidal antiandrogen flutamide, which exerts its effects by interfering with the binding of dihydrotestosterone or testosterone to the androgen receptor (5).

The study of prostate cancer chemoprevention has been hindered by the lack of appropriate animal models. Recently, a unique animal model known as the TRAMP model of prostate cancer has been described (6, 7). In TRAMP mice, targeted expression of Tag driven by the prostate-specific promoter PB leads to transformation of cells in the prostate. This animal model has several advantages over the currently existing models: (a) the tumors occur with 100% frequency; (b) the mice develop prostatic epithelial hyperplasia and PIN, a premalignant lesion, as early as 10 weeks and develop invasive adenocarcinoma around 18 weeks of age; (c) the mice spontaneously develop invasive primary tumors that metastasize to the lymph nodes, lungs, and bone in a pattern similar to that of human prostate cancer; and (d) the development and progression of prostate cancer can be followed within a relatively short period of 10–30 weeks. The ability to identify animals predestined to develop prostate cancer and modify their environment may allow for the expedited evaluation of potential chemopreventive agents.

Using the TRAMP animal model, a pilot study was conducted to test the efficacy of flutamide in the prevention of prostate cancer. Here we report that flutamide has the ability to significantly suppress prostate carcinogenesis as evidenced by a longer latency period of prostate cancer formation and a lower incidence of prostate cancer in the TRAMP model.

MATERIALS AND METHODS

A pilot study was undertaken to test the efficacy of flutamide in the TRAMP transgenic animal model, in which every animal that inherits the transgene develops prostate cancer. The animal experimental protocol was approved by an institutional animal experimentation review board and followed NIH guidelines for proper and humane use of animals. PB-Tag transgenic C57BL/6 mice were cross-bred with FVB wild-type strain mice, the hybrid litters were screened for positive animals, and the drug dose was adjusted for growth-related changes in weight. The protocols implanted s.c. through a 1-cm incision on the flank into PB-Tag mice (30 days of age; average age, 14 g) anesthetized with ketamine (Mallinckrodt, Mundelein, IL). Three groups of 10–15 animals each received

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1 To whom requests for reprints should be addressed, at University of Tennessee Medical Center, Department of Urology, 956 Court Avenue, F210, Memphis, TN 38163. Phone: (901) 448-2636; Fax: (901) 448-4743; E-mail: sraghow@utmem.edu.

2 The abbreviations used are: TRAMP, transgenic adenocarcinoma of the mouse prostate; Tag, T antigen; PB, probasin; PIN, prostatic intraepithelial neoplasia; TGF-β1, transforming growth factor β1.
FLUTAMIDE AS A CHEMOPREVENTIVE AGENT

RESULTS

The high dose of flutamide decreased the incidence and increased the latency of prostate cancer. Pulpable tumor formation was not significantly different between the low-dose flutamide and placebo groups. In both of these groups, tumors initially presented at 17 weeks of age, and by 33 weeks of age, all of the animals had developed palpable tumors.

Table 1  Statistical analysis

<table>
<thead>
<tr>
<th>Test</th>
<th>Placebo vs. low-dose flutamide</th>
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<td>Log-rank (P)</td>
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<td>Wilcoxon’s rank test (P)</td>
<td>0.8628</td>
<td>0.0036</td>
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a P < 0.05 level of significance.

A 90-day-release drug pellet of either a low dose of flutamide (6.6 mg/kg) or a high dose of flutamide (33 mg/kg) or a placebo (a pellet with no pharmacological activity). Each treated animal received supplemental dosages at 90-day intervals until tumors were palpable. The efficacy of the treatment was measured by the absence of a palpable tumor. Starting at 10 weeks of age, animals were evaluated weekly for the presence of a palpable tumor, the end point of the study. Mice were euthanized with carbon dioxide, and necropsy examination was performed to confirm the presence and origin of the tumor. The statistical analysis compared the differences between treatment groups by Fisher’s exact test and Wilcoxon’s rank test (8). All P values were two-sided.

Whole Mounts and Histology. Ventral prostate lobes from representative animals in the placebo-treated and high-dose flutamide-treated groups were resected at 7, 10, 15, and 20 weeks for examination under dark-field microscopy using the Olympus SZH stereo-dissection scope fitted with an Olympus camera. Murine prostate tissues/tumors were harvested, fixed overnight in 4% paraformaldehyde, processed in a Shandon-Lipshaw tissue processor, and embedded in paraffin. Tissue sections (4-μm thick) were stained with H&E for histological evaluation.

Western Blot Analyses. Ten cross-bred Tag-positive male pups (5 per group) were treated with either placebo or flutamide pellets at 4 weeks of age. Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 weeks of age, snap-frozen in liquid N2, and stored at −80°C. Tissue lysates were prepared using radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris (pH 7.5)] containing a mixture of protease inhibitors (Pefabloc, aprotinin, bestatin, leupeptin, and pepstatin) and the phosphatase inhibitor Na3VO4 (10 mM). The homogenate was centrifuged at 14,000 g at 4°C for 10 min, and lysates were stored at −80°C until use.

Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Tissue lysates were loaded onto 7.5% polyacrylamide gels, and proteins (40 μg/lane) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (0.2 μm; Bio-Rad) using a transfer buffer (192 mM glycine, 25 mM Tris-HCl, and 20% methanol). TRAMP prostate tumor tissue was used as a positive control. Chemiluminescent Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA) were used as molecular weight standards. Blots were blocked overnight at 4°C in BLOTTO (6% nonfat dry milk in 1× TBS) and incubated with the large Tag primary antibody (Pab 101 mouse monoclonal antibody; 1:200; Santa Cruz Biotechnology) for 2 h at room temperature. The blots were washed three times with TTBS (0.05% Tween 20, 50 mM Tris-HCl, and 200 mM NaCl) and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at 25°C. Immunoreactive proteins were visualized on autoradiography film using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). Actin protein expression was used to normalize Tag results. For this purpose, the above-mentioned membrane was submerged in stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7)] and incubated at 50°C for 30 min with occasional agitation. After blocking, the membrane was reprobed with actin primary antibody (1:2,500; Chemicon, Temecula, CA), followed by horseradish peroxidase-conjugated secondary antibody (1:10,000). After enhanced chemiluminescence detection, band intensities were quantitated using the Adobe Photoshop 5.0 Acquisition and ImageQuant Analysis (Molecular Dynamics) systems.

Fig. 1. Chemopreventive effects of flutamide in the TRAMP model. Transgenic mice were divided into three groups: (A) placebo; (B) low-dose flutamide (6.6 mg/kg/day); and (C) high-dose flutamide (33 mg/kg/day). Starting at 10 weeks of age, animals were examined weekly for the presence of a palpable tumor. Each point represents the number of animals without palpable tumors (percentage tumor free) in the Kaplan-Meier graph.

Fig. 2. Effect of flutamide on prostate tumor development in the TRAMP model. Dark-field microscopy of ventral prostate whole mounts showing prostatic ducts joining the urethra. A – D, placebo-treated prostate; E – H, high-dose flutamide-treated prostate.

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palpable tumors. In the high-dose flutamide-treated group, however, tumors were not palpable until 24 weeks of age, a lag of 7 weeks, and by 34 weeks of age, 42% of the animals had no palpable tumors (Fig. 1). The period of time at which 50% of the animals had tumors was 33 weeks in the high-dose flutamide group, 24.5 weeks in the low-dose flutamide group, and 24.5 weeks in the placebo group. The end point in this pilot study was a palpable tumor. Therefore, although two animals in the high-flutamide group were tumor free at 38 weeks, the study was terminated because all animals in the other two groups had developed tumors. The difference between the placebo and high-dose flutamide groups was statistically significant by both log-rank and Wilcoxon analysis with a \( P \) of 0.0036 and 0.0060, respectively (Table 1).

The cancer-inhibitory effect of flutamide, using a palpable tumor as the end point, was substantiated by whole mount analysis of prostate tissue of representative animals from the placebo-treated and the high-flutamide-treated groups (Fig. 2, \( A-D \) and \( E-H \), respectively).

Histological examination of the mouse prostate tissue revealed that the normal prostate was replaced by sheets of undifferentiated, anaplastic cells in the 17-week-old TRAMP mouse prostate. PIN was observed in the prostate tissues of 15-week-old, placebo-treated animals. However, prostate of the comparable 15-week-old, high-dose flutamide-treated animals showed no PIN, and its ductal appearance resembled that of the 17-week-old wild-type prostate (Fig. 3). Tumors from the placebo-, low-dose flutamide-, and high-dose flutamide-treated groups were harvested 6 weeks after they became palpable. Microscopic examination of the tumor tissue histology from placebo-treated animals showed that the normal prostate (Fig. 4A) was replaced by sheets of undifferentiated, anaplastic cells with a high mitotic index (Fig. 4B). Tumors from the low-dose flutamide-treated group (Fig. 4C) were similar to those of the placebo-treated group. In contrast, the high-dose flutamide-treated mice (Fig. 4D) had tumors that were distinctively differentiated and retained a glandular architecture; the mitotic index was much lower than that of the placebo-treated group. Thus, flutamide treatment significantly decreased the incidence of prostate cancer and increased the latency period of prostate cancer in TRAMP mice. Moreover, mice treated with high-dose flutamide had more differentiated tumors.

The effect of flutamide treatment on Tag expression was determined in duplicate by Western blot analysis, and representative data are shown in Fig. 5. Tag was present in the prostate tumor tissue resected at 24 weeks age. The oncoprotein was also present in tissues of 10-week-old placebo-treated animals. Based on the ratio of Tag:actin (housekeeping protein), flutamide-treated animals expressed significantly lower levels of the Tag than did the comparable placebo-treated animals (Fig. 5).

**DISCUSSION**

Hormonal factors appear to play an important role in the development of prostate cancer because eunuchs do not have prostate cancer, and prostate cancer can be induced in Noble rats by the chronic administration of testosterone (9, 10). Androgens regulate prostatic epithelial proliferation by modulating stimulatory and inhibitory growth factors to maintain homeostasis.

Because androgen promotes carcinogenesis, its inhibition remains a logical first approach for prostate cancer prevention. Gingrich et al. (11) examined the consequences of androgen deprivation by castration on the initiation of prostate cancer and progression to metastatic prostate cancer in TRAMP mice. Their studies revealed that although castration at 12 weeks age significantly reduced the genitourinary tumor burden, the overall progression was not ultimately delayed, and tumors that did develop were always poorly differentiated. In fact, Ferguson et al. (12) reported a marked decrease in the prevalence and extent of high-grade intraepithelial neoplasia in the prostates of patients receiving androgen deprivation therapy compared with the prostates of untreated patients. Finasteride, a 5α-reductase inhibitor, is currently being investigated as an agent to prevent prostate cancer in the National Cancer Institute-sponsored Prostate Cancer Prevention Trial. However, its ability to prevent prostate cancer in animals has
never been demonstrated. Consequently, other agents with demonstrable efficacy against prostate cancer oncogenesis should be explored.

We believe that the present study used a better model (5) and a more reliable drug delivery method than the previous prostate cancer chemoprevention studies (13). The slow-release s.c. implanted pellets provide a more controlled and more reliable drug dosage than the conventionally used ad libitum diet method, which may introduce significant variability. Using the approach in our study, the high-dose flutamide treatment increased the latency period of prostate cancer by 7 weeks. Thus, the disease was significantly (7/24 = 29%) delayed.

Moreover, the tumors were more differentiated in the 42% of the mice that ultimately developed prostate cancer. Histological examination showed that tumors from high-dose flutamide-treated animals were more glandular in architecture compared with those of the placebo group, suggesting that flutamide was able to interfere with tumor progression. These results are in direct contrast to the castration data by Gingrich et al. (11), where 65% of the castrated animals developed tumors, and 100% of tumors were poorly differentiated. In the TRAMP model, the early events leading to carcinogenesis are in effect long before the 10 weeks age, when the mice develop preneoplastic lesions (5). Thus, a major difference between the two studies is the timing of androgen deprivation, i.e., early androgen deprivation at 4 weeks age (this study) versus castration at 12 weeks age (5).

These data imply that androgen ablation with flutamide during the early stage of carcinogenesis may be an effective chemopreventive measure against prostate cancer. It is conceivable that castration sets up an environment conducive to more aggressive androgen-independent disease. The observation that titration of androgen by flutamide was less severe than castration suggests the presence of additional androgen receptor-mediated signals that are not blocked by flutamide and enable the cells to maintain a more differentiated phenotype. Interestingly, overexpression of TGF-β1 has been shown to reduce mammary tumor formation in transgenic mice. This raises the possibility that agents able to stimulate TGF-β1 production/activity may also prevent other hormone-responsive tumors like prostate cancer (14–17). Flutamide has been shown to stimulate TGF-β1 production in regressed human prostate cancer (18) and induces the involution of rat normal prostate (8). This suggests that the chemopreventive effects of flutamide might be mediated through TGF-β1.

In addition to the notable delay, the significant decrease in prostate cancer incidence suggests that flutamide at a higher dose may be an effective chemopreventive agent. Earlier experiments in rats had calculated the minimum effective antiandrogen dose for flutamide to be 5 mg/kg body weight/day (5). Later studies on rats, dogs, and baboons used flutamide at 50 mg/day, which was 10 times the minimum effective dose (5, 19). Because a flutamide dose of 6.6
According to Simard et al. (20), who studied the interaction of flutamide with the androgen receptor in the rat ventral prostate and in human prostatic carcinoma, higher concentrations of antiandrogens were needed to efficiently prevent androgen receptor binding by androgen.

Flutamide exerts its antiandrogen influence by blocking ligand binding to the androgen receptor (5). It appears that in the TRAMP model, this antiandrogen influence is conferred upon and results in the decreased expression of the p53 protein for its assigned role as the gatekeeper of cellular growth and division (21), which results in the delay of prostate cancer. The SV40 large Tag binds and inactivates p53 protein (22), and the loss of tumor suppressor wild-type p53 and Rb genes has been implicated in the development of prostate cancer (23, 24). In the TRAMP model, Tag expression leads to abrogation of p53 and Rb functions, predispersing these cells to genetic instability. In this regard, the TRAMP model is significantly different from human prostate cancer, in which p53 and Rb come into play at a much later stage. However, carcinogenesis in the TRAMP model is primarily androgen driven, it provides a very sensitive system to measure the consequence of hormone ablation in an in vivo model and assess the efficacy of potential androgen analogues.

Flutamide, at the effective high dose (33 mg/kg/day) used in our study, was well tolerated in these animals, with no obvious signs of toxicity. In human studies, the toxicity profile of flutamide, unlike retinoic acids, is reportedly favorable (26). Using the accepted algorithm (26), this translates into 165 mg/day as a chemopreventive dose for treatment of prostate cancer. Finally, flutamide works at the prostate level; consequently, testosterone blood levels are not reduced, and libido and potency are maintained (27). This is critical because men without overt prostate cancer will only be interested in taking chemopreventive agents with a low toxicity profile. Thus, we believe that flutamide is an antiandrogen with a potential for use in clinical prostate cancer chemoprevention trials.

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Efficacious Chemoprevention of Primary Prostate Cancer by Flutamide in an Autochthonous Transgenic Model

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