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TITLE: Role of the TGF-β 1 in the Prevention of Prostate Cancer

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Role of the TGF-β 1 in the Prevention of Prostate Cancer

Our preliminary data showed that antiandrogen (toremifene) and antiestrogen (flutamide) prevented cancer in the TRAMP transgenic model. We hypothesized that these agents inhibit prostate carcinogenesis through stimulation of TGFβ production. This hypothesis is being tested through two specific aims: 1) whether the chemopreventive biologic effects of antiandrogens, antiestrogens and retinoic acid are mediated by TGFB in the TRAMP model, 2) whether prostate cancer may be prevented in the TRAMP model at the genetic level by crossbreeding with transgenic mice engineered to overexpress TGFB. While the retinoid MDI301 was ineffective, both flutamide and toremifene were able to delay onset of prostate cancer. The mechanism of this suppression may be different for the two agents: flutamide inhibited but toremifene did not affect large T-antigen expression. Toremifene treated animals had higher total and free testosterone levels. However, androgen receptor levels were similar for placebo and toremifene treated animals. Since toremifene inhibited prostate cancer in a milieu of elevated free testosterone levels the mechanism of toremifene chemopreventive activity appears to be through nonandrogenic pathways. One potential mechanism may be through stimulation of TGFβ. TGFB overexpression in the prostate or seminal vesicles delayed tumor development in the TRAMP mice through autocrine and paracrine pathways.
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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in men, and the frequency of prostate cancer increases with each subsequent decade of life. Although prostate cancer is initially androgen-dependent, it usually progresses to the hormone-refractory advanced disease, for which there is no cure. The high incidence of this disease and its associated mortality make it imperative to develop prevention strategies against this disease.

Modifications in environmental, dietary, endocrine, or genetic factors may play a role in the prevention of prostate cancer. Within the cellular microenvironment, peptide growth factor TGFβ has the ability to inhibit normal epithelial cell growth suggesting that agents that can stimulate TGFβ production by prostate cells may prevent clinical prostate cancer. Unfortunately, it has been difficult to test this hypothesis as the study of prostate cancer chemoprevention has been hindered by the lack of appropriate animal models. Recently, a unique animal model, known as the transgenic adenocarcinoma of the mouse prostate (TRAMP), of prostate cancer has been described. In TRAMP mice, targeted expression of T antigen (Tag) driven by the prostate specific promoter probasin (PB) leads to transformation of cells in the prostate. Our preliminary studies have focused on three classes of agents that are known to stimulate TGFβ in prostate cells: antiestrogen, antiandrogen, and retinoic acid. Our preliminary data have revealed that antiestrogen (toremifene) and antiandrogen (flutamide) had the ability prevent prostate cancer in the TRAMP transgenic mouse model. The exact mechanism of prostate cancer prevention by these agents, however, is unclear. We hypothesized that these agents stimulate TGFβ production that in turn inhibits prostate carcinogenesis by preventing the activation of latent prostate cancer. This hypothesis is being tested in TRAMP transgenic mice, which develop spontaneous prostate cancer with features similar to that of human prostate cancer, through two specific aims. 1) To determine whether the chemopreventive biologic effects of antiandrogens, antiestrogens, and retinoic acid are mediated by TGFβ1 in the TRAMP model, and 2) To investigate whether prostate cancer may be prevented at a genetic level in the TRAMP model by cross breeding with transgenic mice that have overexpression of TGFβ1 in the prostate. Hence, identification of a chemopreventive agent with proven biologic efficacy in an exciting new prostate animal model with appropriate surrogate markers of carcinogenesis should have important implications for human prostate cancer chemoprevention clinical trials.
REPORT BODY

Task 1: To determine whether the chemopreventive biologic effect of antiandrogens, antiestrogens, and retinoic acid is mediated by TGFβ1 in the TRAMP model

To characterize the relative chemopreventive efficacy of chemopreventive agents (months 1-12)

1. Breeding, screening, and pellet implantation (months 1-3)
   i. Antiandrogen (flutamide 30mg/kg/day, 50 TRAMP mice)
   ii. Antiestrogens (toremifene 10mg/kg/day, 50 TRAMP mice)
   iii. Retinoic acid (9cis RA 1mg/kg/day, 50 TRAMP mice)
   iv. Control (Placebo pellets, 50 TRAMP mice)

2. To evaluate the morphometric changes of the prostate associated with chemoprevention (months 3-15)
   i. Computer assisted morphometric analysis of histology (% epithelium &% stroma)
   ii. Wholemount prostate dissections

3. To evaluate changes in serum androgens and estrogens with chemoprevention (months 1-12)

4. To assess the molecular changes responsible for chemoprevention (months 6-18)
   i. Androgen receptors immunohistochemistry
   ii. TGFα, EGFR, TGFβ1-3 and TGFβ receptors RI and RII semiquantitative RT-PCR
   iii. TGFα, EGFR, TGFβ1-3 and TGFβ receptors RI and RII immunohistochemistry
   iv. TGFα, EGFR, TGFβ1-3 and TGFβ receptors RI and RII in situ hybridization
   v. Differentiation status – vimentim and cytokeratins immunohistochemistry

5. To determine which intermediate biomarkers of prostate cancer correlate with the transformation of latent to clinical prostate cancer and as a consequence may be used as surrogate endpoints for studying chemoprevention:
   i. Prostate intraepithelial neoplasia (PIN) grading (months 12-18)
   ii. DNA repair enzymes assays (months 18-24)
   iii. Telomerase activity assays (months 18-24)
   iv. Peptide growth factor and growth factor receptor expression for TGFα and TGFβ1-3 (as above)

Task 1 Status

We have tested the following agents for chemopreventive activity against prostate cancer in the TRAMP model: antiandrogen (flutamide), antiestrogen (toremifene) and retinoid (cis-retinoic acid derivative MDI301). Our studies indicate that both antiandrogens and antiestrogens exhibit chemopreventive activity in the TRAMP model. These studies were published in, "Efficacious Chemoprevention of Primary Prostate Cancer by Flutamide in an Autochthonous Transgenic Model " by Raghow et al. Cancer Res. 60: 4093-4097, 2000,” and Toremifene prevents Primary Prostate Cancer in the TRAMP transgenic model" by Raghow et al. Cancer Research (submitted, copy of manuscript attached). Palpable tumors appeared in the placebo-treated animals by 15 weeks age, and by 30 weeks 100% animals had tumors compared with 57% of flutamide-treated and 28% of toremifene-treated animals (Table 1). The chemopreventive delay of prostatic tumors by flutamide and toremifene beyond 20 weeks was also quite apparent. The seminal vesicle size in the drug-treated animals was much smaller compared to the placebo, the effect of toremifene being much more pronounced than that of flutamide (Fig. 3). However, the MDI301 used in this study showed no chemopreventive effect (Fig. 1). The MDI301-treated animals had prostate tumors
at the same time or even earlier than the placebo group (10 weeks vs. 15 weeks of age in the placebo (Fig. 1A) and no change in the seminal vesicle size compared to the placebo (Fig. 1B). Consequently, further retinoic acid treatment and sampling was halted and, thereafter, the study focused on the flutamide and toremifene groups.

To conduct these studies, hybrid TRAMP mice (C57/BL6-PBTag x FVB wildtype) litters were screened for the SV_{40} large T-antigen (Tag) transgene and the positive males were implanted with the placebo or flutamide (33mg/kg/day) or toremifene (10mg/kg/d) pellets at 4 weeks age. During the first 12-18 month period, the emphasis was on the whole mount studies and collecting the appropriate tissues for histology as well as molecular studies. For each time point, 5-10 animals each were treated with either placebo, or flutamide or toremifene or MDI301. Animals were sacrificed at 7, 10, 15, 20, 25 and 30 weeks of the proposed schedule and tissues (ventral prostate, dorsolateral prostate, anterior prostate and seminal vesicles) harvested for morphology (whole mounts), for histology (formalin-fixed, paraffin-embedded), and molecular studies (frozen in liquid N_{2} and stored at -80°C). Blood was collected and the pooled serum was stored frozen for hormone analyses.

Whole mount studies using dark-field microscopy was done to ascertain the absence/presence of a non-palpable tumor, to locate the origin of the tumor and any change in the ductal development. The ventral prostate, anterior prostate and seminal vesicle whole mounts for 7,10,15, 20 and 25 and 30 weeks for the various groups have been completed. Dissection of the animals for whole mount studies confirmed the time of palpable tumor appearance assessed in our pilot study i.e. palpable tumors in non-treated TRAMP mice appear at 15-20 weeks of age.\textsuperscript{13}\textsuperscript{14}

Next, we studied the histological changes associated with initiation and progression of PIN and its delay by chemopreventive drugs. Since frank tumors in the placebo-treated animals appeared between 15-20 weeks age these samples revealed any signs of PIN and helped narrow down the window of drug efficacy. Parallel studies of the relevant samples are being done to assess molecular changes related to PIN and the drug efficacy. 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 the 15 week-old placebo-treated animals. However, prostate of the comparable 15 week-old high flutamide-treated animals showed no PIN and its ductal appearance resembled that of the 17 week-old wild-type prostate (Fig. 3).\textsuperscript{13} Tumors from placebo, low dose flutamide, and high dose flutamide 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. 4, A)\textsuperscript{13} was replaced by sheets of undifferentiated, anaplastic cells with a high mitotic index (Fig. 4, B).\textsuperscript{13} Tumors from the low dose flutamide-treated (Fig. 4, C)\textsuperscript{13} group were similar to those of the placebo group. In contrast, the high dose flutamide-treated (Fig. 4, D)\textsuperscript{13} mice had tumors that were distinctively differentiated retaining a glandular architecture; the mitotic index was much lower than the placebo group.\textsuperscript{13} Moreover, mice treated with high dose flutamide and toremifene had more differentiated tumors.

A study was also carried out to alleviate a primary but very important concern of the DOD proposal Reviewer A: "Weaknesses are that prostate cancer in the TRAMP model is induced by the expression of SV_{40} large T-antigen under the control of the probasin promoter, which is androgen-dependent and mainly prostate specific. The preventive effects of antiandrogens and/or antiestrogens could be mediated by the inhibition of the SV_{40} T-antigen expression driven by the probasin promoter. No experiment is proposed to address the effects of antiandrogens, antiestrogens and retinoic acids on the SV_{40} T-antigen expression". To answer this question, animals were bred, screened and implanted with placebo, flutamide (antiandrogen) and toremifene
(antiestrogen) pellets. The Western Blot (WB) and the chemiluminescence techniques were optimized and applied to measure the Tag expression in the drug-treated prostate tissue lysates vs. the placebo. The TRAMP tumor tissue was used as the positive control. There was abundance of the T-antigen in the prostate tumor tissue resected at 24 weeks age. The T-antigen oncoprotein was also present in the 10 week-old placebo-treated and flutamide-treated animals, with level in the flutamide-treated animals being significantly lower than in the comparable placebo-treated animals (Fig. 5). These results indicate that the delay/inhibition of cancer initiation by flutamide is most likely mediated through inhibition of the Tag expression by interfering with the androgen-responsive elements of the probasin promoter. The TRAMP model, in this context, is still a valid model for comparing and evaluating the potency of various antiandrogens as chemopreventive agents.

The efficacy of toremifene was significantly higher than of the comparable flutamide doses (Table 1). Histological examination of the mouse prostate tissue revealed PIN in the prostate tissues of the 7 and 15 week-old placebo-treated animals (Fig. 4, A-B) but not in the prostate tissues of comparable 7 and 15 week-old toremifene-treated animals (Fig. 4, C-D). Tumors from the high dose toremifene groups were harvested 6 weeks after they became palpable. Tumors from toremifene-treated mice resembled those from flutamide-treated mice in that they were more differentiated and retained a glandular architecture compared to the placebo-treated mice (not shown). Thus, toremifene treatment significantly decreased the incidence of, and increased the latency period of prostate cancer in TRAMP mice. Interestingly, the Tag levels in toremifene-treated animals were significantly higher than in the placebo-treated animals and it seems that the mechanism of inhibitory effect of antiestrogens on prostate cancer development in the TRAMP does not involve Tag. The Tag oncoprotein was clearly present in the prostate tumor tissue resected at 20 weeks age as well as in the placebo-treated prostate at 15 weeks age (Fig. 6, A). Surprisingly, however, toremifene-treated prostate Tag level was noticeably higher than the placebo-treated prostate. Similar results were obtained with toremifene-treated 10 weeks old prostate tissue (Fig. 6, B) in which Tag expression maintained at levels higher than the comparable placebo-treated prostate tissues.

Serum testosterone and estradiol levels were assayed using the enzyme immunoassay (EIA) kits (DSL-10-4000ACTIVE™) and (DSL-10-4300ACTIVE™), respectively, supplied by Diagnostic Systems Laboratories, Inc. Houston, TX. Values for the sample analytes were determined by interpolation, using standards available with the kit. Flutamide or toremifene treatment did not affect serum estradiol levels, which remained almost unchanged between 10-30 weeks age. Both total and free testosterone levels in serum from flutamide-treated animals also did not differ much from the placebo-treated animals until 25-30 weeks age where it slightly increased. Paradoxically, serum from toremifene-treated animals at 10-20 weeks age showed a sharp increase in both total and free testosterone, the levels of these hormones being approximately 25-fold and 40-50-fold, respectively (Table 2). This finding prompted us to further investigate the hormonal axis and the androgen receptor level. The androgen receptor status in the placebo and toremifene-treated prostate tissues was analysed by Western blot. The hybrid TRAMP (TRAMP x FVB) tumor tissue had much higher level of androgen receptor than the prostate tissues of comparable age nontransgenic mouse of the same genetic background (C57/BL6 x FVB) (Fig. 7). Interestingly, the prostatic androgen receptor levels were similar for placebo and toremifene treated TRAMP mice and resembled that from the tumor tissue. Thus toremifene treatment did not significantly affect the androgen receptor expression.
Tamoxifen has been previously shown to down regulate androgen receptor expression as one of the mechanisms that a SERM could suppress androgen dependent tissues. The seminal vesicles, like the prostate, are androgen-dependent. Consistent with this possible mechanism, toremifene inhibited androgen dependent seminal vesicle development in the presence of elevated serum free testosterone levels suggesting that toremifene appears to be acting as an antiandrogen. However, many of our experimental observations do not support this antiandrogenic mechanism: 1) Toremifene did not suppress the probasin promoter which contains an androgen response element (ARE), 2) The size of the prostate glands were similar for the toremifene and placebo treated animals prior to 15 weeks of age, 3) Prostatic androgen receptor levels were similar for toremifene and placebo treated TRAMP mice, and 4) Prostate cancer formation was inhibited in a milieu of elevated free testosterone levels. Thus, the mechanism of toremifene chemopreventive activity appears to be through nonandrogenic pathways. In fact, toremifene was a more potent chemopreventive agent than antiandrogen flutamide.

The toxicity profile of both flutamide and toremifene in the TRAMP mice was quite favorable. Toremifene has been demonstrated to be as effective as tamoxifen against breast cancer, but is less uterotrophic than tamoxifen in the rat model. Toremifene treatment reduced incidence of mammary tumors in females and testicular tumors in male rat. Tamoxifen increases the risk of uterine and endometrial cancer due to DNA-adduct formation. This DNA reactive property also leads to liver toxicity in rats. While carcinogenicity of the uterus and endometrium is not applicable to the prostate cancer, subjects being male, we looked for signs of cancer of other related organs such as testis, epididymis, vas deferens, seminal vesicles, and bladder. Both flutamide and toremifene at the effective high dose (33mg/kg/day) used in our study, were well tolerated and the TRAMP mice did not show any adverse effects on these organs during the course of the treatment.

Using the accepted algorithm, this dose translates into 165 mg/day as a chemopreventive dose for human subjects.

The chemopreventive mechanism of toremifene is still unclear. Recent in vitro studies have demonstrated that tamoxifen can induce the autocrine secretion of TGFβ in human breast cancer cells resulting in the inhibition of cellular growth. Tamoxifen has also been reported to induce the secretion of active TGFβ from human fetal fibroblasts despite the absence of ER within these cells. Interestingly, overexpression of TGFβ1 has been shown to reduce breast cancer tumor formation in mice raising the possibility that TGFβ stimulating agents may also prevent other hormone responsive tumors like prostate cancer. In vivo, induction of extracellular TGFβ1 in the stroma of human breast tumors as early as 3 months of tamoxifen treatment indicated tamoxifen inhibition through an ER-independent mechanism. In rat, toremifene exerts multiple effects on a variety of genes involved in the control of signaling and apoptosis, by causing distinct changes in steroid receptors, p53, and bcl-2 expression. Estrogens and antiestrogens influence the G1 phase of the cell cycle. In MCF-7 breast cancer cells, estrogen stimulated cell cycle progression through loss of the kinase inhibitory protein p27 and p21 and through G-1 cyclin-dependent kinase (cdk) activation. Depletion of either p21 or p27 by antisense can mimic estrogen-stimulated cell cycle activation and indicate that both proteins are critical mediators of the therapeutic effects of antiestrogens in breast cancer. Tamoxifen inhibition of prostate cancer cells in preclinical studies was associated with inhibition of protein kinase C and direct activation of the TGFβ signaling pathway, including induction of p21 and p16.

The molecular mechanism of action of toremifene is currently being investigated. Currently, RT-PCR and Western blot methods for TGFβ (Fig. 2) and the receptors (Fig. 3) have been optimized and sample analyses is underway. Our preliminary data shows that TGFβ1 (Fig. 2, a) and TGFβ3 (Fig. 2, C), but not TGFβ2 (Fig. 2,
B), are down regulated in the TRAMP mice. Expression of both receptors TGFβRI (Fig. 3, A) and TGFβRII (Fig. 3, B) is also inhibited. RT-PCR analyses on prostate tissues from placebo and flutamide or toremifene-treated animals at ages 7, 10, 15 and 20-week also indicate involvement of TGFβ pathway in the chemopreventive activity (data being analysed). Immunohistochemical methods for localization of the growth factors and receptors in Raghow et al. "Immunohistochemical localization of Transforming Growth Factor-α and Transforming Growth Factorβ during early human fetal prostate development". Journal of Urology, 1999, 162:509-513 will be used with some modification (reprint attached). Tissues have been collected to apply these techniques. All tissues from all treatment group time points have been collected so that all the tissue sections may undergo immunohistochemistry together. This will minimize the inter- and intra-assay variability.) Levels of p21 in these samples seem to be very low and undetectable by WB (Fig. 4, A) but detectable by immunoprecipitation (IP) (Fig. 4, B). TGFβ is detectable only in MMTV-TGFβ seminal vesicles (Fig. 4C) by WB. Alternatively, immunohistochemical method for TGFβ and p21 are being optimized using the sensitive signal enhancement technique and may be used for quantitation.

Additionally, ERα was significantly inhibited in the TRAMP prostate tissues compared to non-transgenic littermates and neither flutamide nor toremifene could reverse this inhibition. In addition to the classic estrogen receptor alpha (ERα), the discovery of a novel estrogen receptor beta (ERβ) in the rat, mouse and human prostate has added a new dimension to understanding of chemopreventive mechanism of antiestrogens. Consequently, the methodology to evaluate both ERα and ERβ expression in prostate tissues is being developed.

**Task 2:** To investigate whether prostate cancer may be prevented at a genetic level in the TRAMP model by crossbreeding with transgenic mice that have overexpression of prostate TGFβ1.

To characterize the chemopreventive efficacy of TGFβ1 overexpression in TRAMP x PB-TGFβ crossbred transgenic mice.

1. Crossbreed TRAMP X PB-TGFβ1 mice and screen by PCR (months 8-14)
2. Compare 50 TRAMP mice, 50 PB-TGFβ1 mice, and 50 TRAMP-PB-TGFβ crossed mice as follows (months 12-24):
3. Evaluate the histologic and morphometric changes of the prostate associated with chemoprevention.
   (months 8-24)
   i. Computer assisted morphometric analysis of histology (% epithelium &% stroma)
   ii. Wholemount prostate study

**Task 2 Status**

Characterization of the PBTGFβ transgenic mice, engineered in our laboratory was done to evaluate the target-specificity of the transgene expression. Of the 7 transgenic mice (5 males and two females) obtained after microinjection, the females died in quarantine. The progeny of the remaining five males was screened by RT-PCR (Fig. 5, T2353-A630, T2371-A634, T2375-A631, and T2377-A650; Fig. 6, T2376-A635; Fig. 7, female progeny of T2371-A642). Table 3 presents a summary of the tissue-specific TGFβ expression. It was observed that line T 2353 showed ventral prostate specific expression of TGFβ, with either negligible or no expression in other organs such as seminal vesicles, anterior prostate, bladder, testis, kidney, liver, spleen, lung, heart and thymus and smaller prostate compared to their littermate nontransgenic males (Fig. 8). This Founder
Line, representing the desired phenotype, was selected for our future experiments. RT-PCR on the prostate tissues of the T2353-F2-generation pups (Fig. 9, A687, A700) was then performed to confirm these observations on the specificity of the transgene expression. These RT-PCR results were then substantiated with whole mount analysis of the ventral prostate, anterior prostate and the seminal vesicles of a 7-week old transgenic A709 vs. a nontransgenic littermate NT-6 (Fig. 10). The prostate specific expression of the TGFβ transgene was associated with reduction in the number of ductal glands and the size of the prostate and the effect was even more pronounced in PB-TGFβ homozygous mice obtained by inbreeding heterozygous mice (Fig. 11). A manuscript Raghow S and Steiner M. Prostate-targeted overexpression of TGFβ in a transgenic mouse model is in preparation.

RT-PCR on the prostate tissues of the Line T 2353 that showed TGFβ expression in ventral prostate but not in other organs and had smaller prostate compared to same age nontransgenic mice and was selected for our future experiments. The RT-PCR results were then substantiated with whole mount analyses of the ventral prostate, anterior prostate and the seminal vesicles. The prostate-specific expression of the TGFβ transgene was associated with reduction in the number of ductal glands and the size of the prostate. These mice were crossbred with the PB-Tag mice to test whether prostate cancer may be prevented at a genetic level in the TRAMP model by cross breeding with transgenic mice that overexpress TGFβ1 in the prostate. In addition, we crossbred TRAMP with MMTV-TGFβ mice (seminal vesicles-targeted TGFβ expression) to study the paracrine effect of TGFβ overexpression on the process of carcinogenesis. Bigenic males expressing both Tag and TGFβ transgenes were followed for further study according to guidelines in Phase I-Task 2. Fifty bigenic males in each group (TRAMP x PB-TGFβ) or (TRAMP x MMTV-TGFβ) are being followed. In this ongoing study, we currently have the data for 10, 15 and 20 weeks age and 25 and 30 week data will follow in time. At each time point 6-9 animals were sacrificed to ascertain the presence of a tumor. The issues were harvested for histological and molecular analyses. None of the groups had tumors before 10 weeks age. At 15 weeks age, all the TRAMP x MMTV-TGFβ animals were still tumor-free while 44% of TRAMP x PB-TGFβ animals developed tumors compared with 72% of the control animals (TRAMP x FVB). At 20 weeks age 100% of control animals had tumors compared with only 33% of TRAMP x PB-TGFβ and only 17% of TRAMP x MMTV-TGFβ. The data is shown in Table 4 and Graph 1. The results were significant by Fisher's Exact Test, with P values of 0.0223 and 0.008 at 15 and 20 weeks, respectively. The results show that TGFβ was able to significantly suppress prostate epithelial cell proliferation and inhibit/delay tumor development by both autocrine (in TRAMP x PB-TGFβ) and paracrine (in TRAMP x MMTV-TGFβ) pathways.

Histological and molecular studies on these samples will follow the guidelines in Task 1. The tissues were fixed, embedded in paraffin, and are being sectioned for H&E stain. Heeding the critique of Reviewer B as to the relevance of DNA repair enzymes assays (months 18-24) and Telomerase activity assays (months 18-24) to this project, we have decided to omit these assays. This will enable us to better focus on the cytokine component and TGFβ signaling pathway intermediates such as p21.
KEY RESEARCH ACCOMPLISHMENTS

* Hybrid TRAMP (TRAMP x FVB) mice palpable prostate tumors first appear between 10-15 weeks age and 100% of animals have tumors by 20 weeks age

* Confirmed by detailed wholemount and histologic analyses that both flutamide (antiandrogen) and toremifene (antiestrogen) were able to delay onset of prostate cancer

* Retinoic acid (cis -Retinoic acid derivative MDI-301) did not inhibit the onset of prostate cancer and as such did not demonstrate chemopreventive activity

* Toremifene showed higher efficacy than flutamide.

* The mechanism of this suppression of prostate cancer may be different for the two agents: flutamide inhibited large T antigen expression, whereas toremifene had no effect on large T antigen expression

* Prostatic androgen receptor levels were similar for toremifene and placebo treated TRAMP mice, and prostate cancer formation was inhibited in a milieu of elevated free testosterone levels. Thus, the mechanism of toremifene chemopreventive activity appears to be through nonandrogenic pathways

* The toxicity profile of both flutamide and toremifene in the TRAMP mice was favorable

* Transgenic mice engineered to overexpress prostatic TGFβ had smaller prostates

* In the ongoing study, TGFβ overexpression in the prostate (PB-TGFβ) or seminal vesicles (MMTV-TGFβ) delayed tumor development at 15 and 20 weeks age

* TGFβ is able to delay onset of prostate cancer through both autocrine and paracrine pathways

REPORTABLE OUTCOMES

1. Animal model: Generation and characterization of the PB-TGFβ mouse model.

2. Abstracts:

3. Manuscripts:
Steiner, MS. and Raghow, S. Review: Antiestrogens for the chemoprevention of prostate cancer. J of Urology (J. of Urology-accepted)

Raghow, S. and Steiner, M. Prostate-targeted expression of TGFβ in a transgenic mouse model (in preparation).

4. **Clinical translational research**: Human Clinical Trial, Phase II pilot study to test the efficacy of chemopreventive agent (Toremifene) in prostate cancer. Co-P I: Sharan Raghow, Ph.D.

**CONCLUSIONS**

The TRAMP animal model represents the first reliable model of prostate cancer. These animals have progression of prostate cancer that mirrors human disease. Chemoprevention seeks to inhibit carcinogenesis and suggests that prostate cancer may be prevented. Using three classes of agents suggested to possess chemopreventive activity, the TRAMP model showed that retinoic acid was ineffective but both flutamide and toremifene suppressed prostate cancer. The mechanism of this chemopreventive action may be different for each of these agents as flutamide treatment resulted in downregulation while toremifene did not affect the hormone responsive PB promoter in the TRAMP model. While serum estradiol levels remained unchanged toremifene treated animals had higher total and free testosterone levels but interestingly the androgen receptor levels were similar for placebo and toremifene treated animals. Since toremifene inhibited prostate cancer in a milieu of elevated free testosterone levels the mechanism of toremifene chemopreventive activity appears to be through nonandrogenic pathways. One potential mechanism may be through stimulation of TGFβ. Toremifene was a more potent chemopreventive agent than flutamide. The implications of this work is that prostate carcinogenesis may be inhibited resulting in a decreased incidence of prostate cancer. Due to their limited toxicity flutamide and toremifene should be considered for human prostate chemopreventive Clinical Trials.

**REFERENCES**


Fig. 1A. Effect of Retinoic acid vs. Placebo on the Ventral Prostate development in the TRAMP mouse.

Fig. 1B. Effect of eRA MDI 301 vs. the Placebo on Seminal Vesicle Development in the TRAMP mouse.
Fig. 2. RT-PCR: (A) TGFβ1, (B) TGFβ2 and (C) TGFβ3 expression in the TRAMP mouse
Fig. 2. RT-PCR: (A) TGFβ1, (B) TGFβ2 and (C) TGFβ3 expression in the TRAMP mouse

Fig. 4 C. TGFβ Western Blot
Figs. 3. RT-PCR: (A) TGFβRI and (B) TGFβRII expression in prostate tissues of non-transgenic and TRAMP mice at 7, 10, 15, 20 and 25 week age. Actin was used as internal control.
Fig. 4 A. p21 Western Blot

Fig. 4 B. p21 Immunoprecipitation
Fig. 5. RT-PCR analyses showing differential TGFβ expression in tissues of transgene-positive pups A630, A631, A634 and A650 from Founder PBTGFβ mice Lines T2353, T2375, T2371 and 2377, respectively.

SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus.

PCR products: 340 bp TGFβ and 460 bp; β-actin (internal control); -ve control, FVB wild-type tissue; +ve control, MMTVTGFβ mouse tissue.
Fig. 6. RT-PCR analyses showing differential TGFβ expression in tissues of transgene-positive pup A635 from Founder mice PBTGFβ-Line T2376. VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus. PCR product 340 bp TGFβ. -ve controls, water and FVB wild-type tissue, +ve control, MMTVTGFβ mouse tissue. Note: Seminal vesicles and anterior prostate were involuted.

Fig. 7. RT-PCR analyses showing differential TGFβ expression in tissues of transgene positive female pup A642 from Founder mice PBTGFβ-Line T2371. OV, ovary; UT, uterus BR, breast; BL, bladder; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus. PCR product: 340 bp TGFβ. -ve control, FVB wild-type tissue, +ve control, MMTVTGFβ mouse tissue.
Fig. 8. Expression of the PBTGFβ gene construct in the non transgenic (-M2) vs. transgenic (A630) progeny of the Founder T2353. Tissue distribution of the TGFβ expression was analyzed by RT-PCR, yielding the 340 bp TGFβ and 460 bp β-actin (internal control) PCR products. PCR -ve control, FVB wild-type tissue; +ve control, MMTVTGFβ transgenic mouse tissue. SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus.
Fig. 9. RT-PCR analyses of tissues from F2 generation pups, A687, A700 of Founder Line T2353 to confirm TGFβ expression. SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate. PCR products: 340 bp TGFβ and 460 bp β-actin (internal control); -ve control, FVB wild-type tissue; +ve control, MMTV TGFβ mouse tissue.
Fig. 10. Dark-field microscopy showing wholemount analyses of Ventral Prostate, Anterior Prostate and Seminal Vesicle from A709 (transgenic PBTGFβ) vs. NT6 (non-transgenic) mouse at 7 weeks age.
Fig. 11. Mouse Ventral Prostate wholemounts at 15 weeks age.
(Nontransgenic vs. heterozygous PBTGFβ vs. homozygous PBTGFβ mouse)
Table 1. Effect of placebo, flutamide or toremifene treatment on incidence of prostate tumor development in the TRAMP model. Three cohorts of animals were treated with either placebo, or flutamide (33 mg/kg/d) or toremifene (10 mg/kg/d) pellets at 4 weeks age and 5-10 animals from each group were sacrificed at 10, 15, 20, 25 and 30 weeks age to examine for presence of tumor.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10-wk</th>
<th>15-wk</th>
<th>20-wk</th>
<th>25-wk</th>
<th>30-wk</th>
<th>33-wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0%(0/10)</td>
<td>50%(4/8)</td>
<td>100%(5/5)</td>
<td>83%(5/6)</td>
<td>100%(7/7)</td>
<td>all died</td>
</tr>
<tr>
<td>Flutamide (33mg/kg)</td>
<td>0%(0/6)</td>
<td>0%(0/10)</td>
<td>43%(3/7)</td>
<td>50%(3/6)</td>
<td>57%(4/7)</td>
<td>*</td>
</tr>
<tr>
<td>Toremifene (10mg/kg)</td>
<td>0%(0/12)</td>
<td>0%(0/9)</td>
<td>14%(1/7)</td>
<td>20%(1/5)</td>
<td>28%(2/7)</td>
<td>43%(3/7)</td>
</tr>
</tbody>
</table>

%=percent of animals with tumor; ( ), actual number of animals, * discontinued.
Table 2. Effect of placebo, flutamide or toremifene treatment on serum testosterone and estradiol levels. Three cohorts of animals were treated with either placebo or flutamide (33mg/kg/d) or toremifene (10mg/kg/d) pellets at 4 weeks age. Animals (5-10) from each group were sacrificed at 10, 15, 20, 25 and 30 week age. Blood from 5 animals was pooled to obtain serum and stored at -20°C for assay of hormone levels. Serum testosterone and estradiol levels were assayed using the enzyme immunoassay (EIA) kits (DSL-10-4000ACTIVE™) and (DSL-10-4300ACTIVE™), respectively, supplied by Diagnostic Systems Laboratories, Inc. Houston, TX. Values for the sample analytes were determined by interpolation using standards available with the kit.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10-wk</th>
<th>15-wk</th>
<th>20-wk</th>
<th>25-wk</th>
<th>30-wk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total testosterone (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.24</td>
<td>0.093</td>
<td>0.27</td>
<td>0.068</td>
<td>0.13</td>
</tr>
<tr>
<td>Flutamide</td>
<td>0.78</td>
<td>0.13</td>
<td>0.022</td>
<td>*</td>
<td>0.047</td>
</tr>
<tr>
<td>Toremifene</td>
<td>5.41</td>
<td>7.8</td>
<td>0.12</td>
<td>0.152</td>
<td>0.001</td>
</tr>
</tbody>
</table>

| **Free testosterone (pg/ml)** |       |        |       |       |       |
| Placebo     | 0.590 | 0.880  | 0.979 | 0.497 | 0.206 |
| Flutamide   | 1.157 | 1.367  | 0.201 | 7.2   | 16.381|

| **Estradiol (pg/ml)** |       |        |       |       |       |
| Placebo     | 37.10 | 17.73  | 23.78 | 38.29 | 30.22 |
| Flutamide   | 37.64 | 37.21  | *     | 35.55 | *     |
| Toremifene  | 39.51 | 36.89  | 48.10 | 36.89 | *     |

* = no sample.
Table 3. TGFβ transgene expression by RT-PCR in tissues of PBTGFβ mice Founder Lines T2353, T2371, T2375, T2376 and T2377.

<table>
<thead>
<tr>
<th>FOUNDER</th>
<th>F1</th>
<th>SV</th>
<th>AP</th>
<th>VP</th>
<th>BL</th>
<th>TS</th>
<th>KI</th>
<th>LI</th>
<th>SP</th>
<th>LU</th>
<th>HT</th>
<th>TH</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2353-M</td>
<td>A630-M</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NT 2-M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>T2375-M</td>
<td>A629-M</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A631-M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>T2376-M</td>
<td>A635-M</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>No SV, AP development</td>
</tr>
<tr>
<td>T2377-M</td>
<td>A650-M</td>
<td>+</td>
<td>RNA?</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>AP, RNA degraded??</td>
</tr>
<tr>
<td>T2371-M</td>
<td>A634-M</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OV</td>
<td>UT</td>
<td>BR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2371-M</td>
<td>A642-F</td>
<td>ovary</td>
<td>uterus+</td>
<td>breast+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NT 20-F</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

M, male; F, female; SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate; BL, bladder; TS, testis; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus; OV, ovary; UT, uterus; BR, breast; ‘+’, TGFβ present; ‘-’, no TGFβ; 0, organ not present.

F1, F1 generation; NT 2-M, nontransgenic male; NT 20-F, nontransgenic female.
Table 4. Effect of overexpressed TGFβ in the prostate (PB-TGFβ) or in seminal vesicles (MMTV-TGFβ) on tumor development in the TRAMP mice at 15 and 20 weeks age.

<table>
<thead>
<tr>
<th>Crossbred strain</th>
<th>Tumor (+)</th>
<th>Tumor (-)</th>
<th>Total</th>
<th>% tumor</th>
<th>Fisher's Exact Test (Pr &lt;= P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (15 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAMP x FVB</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>TRAMP x PB-TGFβ</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>44%</td>
<td>0.0223</td>
</tr>
<tr>
<td>TRAMP x MMTV-TGFβ</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Age (20 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAMP x FVB</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>TRAMP x PB-TGFβ</td>
<td>3</td>
<td>7</td>
<td>10</td>
<td>33%</td>
<td>0.008</td>
</tr>
<tr>
<td>TRAMP x MMTV-TGFβ</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>17%</td>
<td></td>
</tr>
</tbody>
</table>

% Tumors

Graph 1. Effect of overexpressed TGFβ in the prostate (PB-TGFβ) or in seminal vesicles (MMTV-TGFβ) on tumor development in the TRAMP mice at 15 and 20 weeks age.
APPENDICES


IMMUNOHISTOCHEMICAL LOCALIZATION OF TRANSFORMING GROWTH FACTOR-α AND TRANSFORMING GROWTH FACTOR-β DURING EARLY HUMAN FETAL PROSTATE DEVELOPMENT

SHARAN RAGHOW, ELLEN SHAPIRO AND MITCHELL S. STEINER

From the University of Tennessee Urologic Research Laboratories, Memphis, Tennessee, and Department of Urology, New York University School of Medicine, New York, New York

ABSTRACT

Purpose: We investigated the role of peptide growth factors and androgens in the developing human prostate.

Materials and Methods: We performed immunohistochemical staining of prostate tissue sections from human fetuses 9.5, 11.5, 13, 16.5, 18 and 20 weeks in gestation.

Results: The temporal and spatial expression of these growth factors was related to the gestational androgen surge. Before the androgen surge (9.5 to 11.5 weeks) transforming growth factor (TGF)-α, TGF-β1 and TGF-β3 but not TGF-β2 were present in the mesenchyme. The epithelium exhibited no detectable staining for any of the growth factors. During the androgen peak (13 to 16.5 weeks) TGF-β1 decreased and TGF-β2 increased in the mesenchyme, and TGF-α, TGF-β1 and TGF-β3 increased in the epithelium. With declining androgen levels TGF-α, TGF-β2 and TGF-β3 remained unchanged but TGF-β1 increased in the mesenchyme with no change in the tested peptide growth factor levels in the epithelium.

Conclusions: These data suggest that androgens regulate the differential expression of TGF-α and TGF-β, and support a role for peptide growth factors as the direct mediators of androgen action on the mesenchymal and epithelial interactions responsible for prostate development.

Key Words: prostate, growth substances, mesoderm, epithelium, androgens
and temporal expression of a mitogenic peptide growth factor TGF-α and the inhibitory growth factors TGF-β1, TGF-β2, and TGF-β3 in early fetal prostate development (9.5 to 20 weeks of gestation). These studies support the hypothesis that peptide growth factors may be the mediators of androgenic action in mesenchymal and epithelial interactions responsible for early prostate development.

**MATERIALS AND METHODS**

The use of human fetal tissue for this study was approved by the New York University School of Medicine Institutional Review Board. Prostate tissue sections from human fetuses 9.5, 11.5, 13, 16.5, 18 and 20 weeks in gestation was formalin fixed, oriented appropriately and paraffin embedded. The entire prostate glands were serially step sectioned (3 μm thick), and 3 sections from the apex, mid-gland (verumontanum) and base were selected for each age group. Immunohistochemistry was performed on 3 μm tissue sections that were prewarmed at 60°C for 30 minutes, deparaffinized in xylene and hydrated through serial ethanol dilutions (100% to 50%). The sections were incubated with 1.5% hydrogen peroxide in methanol to quench endogenous peroxidase activity.

Following a 30-minute block in 0.5% casein/phosphate buffered saline containing the appropriate normal serum, the samples were incubated for 1 hour with primary antibody (1:1,000 in 0.5% casein/phosphate buffered saline, monoclonal rabbit antibod for TGF-α 153 to 159 amino acids; 1:500 in 0.5% casein/phosphate buffered saline, polyclonal goat anti-human LAP antibody AB-246 PB for TGF-β1; 1:100 in 0.5% casein/phosphate buffered saline, polyclonal rabbit antiparacrine antibody AB-12 NA for TGF-β2, and 1:100 in 0.5% casein/phosphate buffered saline, polyclonal goat antichicken antibody AB-244-NA for TGF-β3. With each experimental run mouse epididymis sections were used as negative (treated with goat or rabbit preimmune serum) and positive (treated with primary antibody) controls. After a thorough phosphate buffered saline rinse (5 minutes X 3 with declined during the androgen surge, again increased in immunostaining intensity (see table 3). There was no change in mesenchymal TGF-α or TGF-β3 but TGF-β1 levels declined during this gestational period (see table). DHT produced in the stroma influenced the epithelium by paracrine pathways and was associated with a distinct increase in TGF-α, TGF-β1 and TGF-β3 but little change in TGF-β2 immunostaining (fig. 3).

Between 18 and 20 weeks of gestation TGF-α, TGF-β2 and TGF-β3 staining remained intense in the mesenchyme (fig. 4, A, B and C). In addition, mesenchymal TGF-β1, which had declined during the androgen surge, again increased in immunostaining intensity (fig. 4, B and table). In the epithelium TGF-α, TGF-β1 and TGF-β3 staining was similar to that observed during the androgen surge (see table). Paradoxically, in the mesenchyme TGF-β1 levels increased with declining androgen levels. The persistence of peptide growth factor levels even with declining androgen levels suggests that peptide growth factors may be the key mediators of continued androgen action during this period of rapid prostate morphogenesis.

**DISCUSSION**

Growth factors have been implicated in benign and malignant growth as possible autocrine and paracrine mediators of growth, and temporal expression of a mitogenic peptide growth factor TGF-α and the inhibitory growth factors TGF-β1, TGF-β2, and TGF-β3 in early fetal prostate development (9.5 to 20 weeks of gestation). These studies support the hypothesis that peptide growth factors may be the mediators of androgenic action in mesenchymal and epithelial interactions responsible for early prostate development.
HUMAN FETAL PROSTATE DEVELOPMENT

FIG. 1. Photomicrographs of mouse epididymis sections immunostained for negative (treated with preimmune serum) and positive (treated with primary antibody) controls for TGF-α (A and B) and TGF-β (C and D). Reduced from ×10.

FIG. 2. Photomicrographs of immunostained histological sections of developing human fetal prostate at 9.5 weeks of gestation for TGF-α (A), TGF-β1 (B), TGF-β2 (C) and TGF-β3 (D). 9.5 weeks, 20×. A, C and D, reduced from ×20. B, reduced from ×10.

stromal and epithelial interactions. Since the precise role of peptide growth factors during different stages of prostatic development is unclear, our immunohistochemical study was undertaken to analyze the expression of the mitogenic growth factor TGF-α and the inhibitory growth factors TGF-β1, TGF-β2 and TGF-β3 in the developing human fetal prostate. Prostatic development is dependent not only on the presence of testosterone, but also on its conversion to DHT. Although testosterone production and Leydig cell hyperplasia begin at 8 weeks of gestation, serum testosterone concentrations peak at about 13 to 16 weeks and gradually decline to female testosterone levels. Evidence from in vitro and organ culture experiments exists that androgens may have only a permissive role, whereas peptide growth factors may be the direct mediators of androgen action. Expression of the enzyme 5a-reductase and conversion of testosterone to DHT during the early phase (11 to 16.5 weeks of gestation) of fetal prostate development are confined to the prostatic mesenchyme and urothelium with no detectable staining in the fetal prostatic epithelial cells. This pattern of expression is similar to human and rat male external genitalia and prostate differentiation which is dependent on local DHT formation early in gestation. Inhibition of 5α-reductase enzyme in the male rat results in feminization of the external genitalia and urethra, and partial inhibition of prostatic development. In man the 5α-reductase deficiency syndrome is characterized by a small or undetectable prostate. Consequently, 5α-reductase and DHT are critical for normal human prostate development. In humans androgen receptor
was initially present in the mesenchyme and urothelium but with the fetal androgen surge the prostatic epithelium had greater androgen receptor staining. This finding suggests that DHT is produced by the androgen receptor positive mesenchyme and affects the androgen receptor positive epithelial cells by paracrine signaling pathways.

Tissue recombinant experiments have demonstrated the critical paracrine relationship between the mesenchyme and epithelium during androgen dependent morphogenesis.\textsuperscript{16,19} In these experiments if the corresponding urogenital mesenchyme lacks androgen receptor as in the testicular feminization syndrome the prostate does not develop, whereas androgen receptor positive urogenital sinus mesenchyme was able to induce androgen receptor negative testicular feminization epithelium to develop into epithelium. Thus, the presence of androgen receptor and DHT is necessary to stimulate mesenchymal elaboration of stromal factors. Our studies show that some of those factors are members of the EGF and TGF-\beta families. TGF-\alpha, TGF-\beta1 and TGF-\beta3 were present in the mesenchyme at significant levels during the period of prostate development before the androgen surge at 9.5 to 11.5 weeks of gestation. In contrast, TGF-\beta2 increased measurably only after 13 weeks, simultaneous with the peak of androgen production by the testes. These observations provide further evidence that mesenchymal DHT stimulates autocrine signaling pathways in androgen receptor positive mesenchyme, which in turn elaborates TGF-\alpha, TGF-\beta1 and TGF-\beta3. It appears that most of the initial changes in androgen and peptide growth factor expression essential for normal human prostate development occur in the mesenchyme.
from 9.5 to 11.5 weeks of gestation. Furthermore, the presence of DHT with the appearance of 5α-reductase and androgen receptor positive epithelium was associated with the highest intensity of immunostaining for TGF-α and TGF-β3 in the epithelium during 13 to 15.5 weeks of gestation. Although TGF-α, TGF-β1 and TGF-β2 are present initially in the mesenchyme, they later appeared in the epithelium during the androgen surge. Hence, it appears that initially DHT only indirectly influences prostatic epithelium by direct induction of mesenchymal factors that diffuse and affect the epithelium in a paracrine fashion.

The level of TGF-β1 was initially high during the early weeks of fetal prostate development but then declined during the androgen surge to the baseline low levels until later when it again increased at 20 weeks of gestation. This reciprocal relationship between presence of DHT and TGF-β1 level seems to suggest down regulation of TGF-β1 by androgens during the period of active fetal prostate development. TGF-β is primarily a growth inhibitor and antagonizes other stimulatory growth factors but not much is known about the differential roles of the specific TGF-β isoforms. Our recent studies showed higher levels of TGF-α and TGF-β3 in the prostatic epithelium during the androgen surge suggesting DHT regulation of this TGF-β isoform in a manner similar to that of mitogenic growth factor TGF-α. TGF-α was initially thought to be produced exclusively by transformed cells but is now known to be present in rapidly growing normal tissues. Overexpression of TGF-α in transgenic mice results in hyperplasia of the anterior prostate. Whereas TGF-α has been shown to be a growth stimulator that may be critical in the cellular proliferation associated with prostate growth, TGF-β3 may be an important factor in continued ductal elongation and morphogenesis. However, the role of TGF-β2 in the developing epithelium remains unclear. Recent studies on rat ventral prostate development reveal that TGF-11, TGF-β2 and TGF-β3 are differentially regulated, that is TGF-β1 and TGF-β2 mRNA expression was enhanced while TGF-β3 mRNA was significantly suppressed after castration. Moreover, the expression of TGF-β2 and TGF-β3 was inversely related. In other experiments TGF-β2 null mice have been shown to have multiple developmental defects including urogenital anomalies with no phenotype overlap with TGF-β1 or TGF-β3 null mice, suggesting distinct regulatory mechanisms and roles for these isoforms.

CONCLUSIONS

The cascade of events starting with the onset of testosterone production by the fetal testes, the expression of functional androgen receptors, and the conversion of testosterone to DHT by 5α-reductase all modulate the differential expression of peptide growth factors in both prostatic mesenchyme and the epithelial cells. These findings support the role of peptide growth factors as local mediators that, by autocrine and paracrine pathways, may be directly responsible for mesenchymal and epithelial interactions leading to prostate development.

REFERENCES

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 antiandrogen activity have become the focus for chemoprevention of this disease. A pilot study was undertaken to test the efficacy of flutamide (an antiandrogen) 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 appeared 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 and (d) 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 expedient 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 by PCR (4) for the presence of the PB-Tag transgene, and only the males that screened positive were used in the study. Flutamide powder was made into slow-release pellets (Innovative Research of America, Sarasota, FL), and the drug dose was adjusted for growth-related changes in weight. The pellets were implanted s.c. through a 1-cm incision on the flank into PB-Tag mice (30 days of age; average weight, 14 g) anesthetized with metofane (Mallinckrodt, Mundelein, IL). Three groups of 10–15 animals each received

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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.
TRAMP prostate tumor tissue was used as a positive control. Chemiluminescent prostate tissue was used as a positive control. Chemiluminescence of prostate tissue was studied using a transfer buffer (192 mM glycine, 25 mM Tris-HCl, and 20% methanol). Dark-field microscopy of ventral prostate whole mounts showing prostatic ducts joining the urethra. A-D, effect of flutamide on prostate tumor development in the TRAMP model. Western Blot Analyses. Ten cross-bred Tag-positive male pups 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.

Table 1. Statistical analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log-rank (P)</th>
<th>Wilcoxon's rank test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose flutamide vs. placebo</td>
<td>0.7955</td>
<td>0.8628</td>
</tr>
<tr>
<td>High-dose flutamide vs. placebo</td>
<td>0.0036*</td>
<td>0.0060*</td>
</tr>
</tbody>
</table>

*P < 0.05 level of significance.

The high dose of flutamide decreased the incidence and increased the latency of prostate cancer. Palpable 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 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 of the study. Mice were euthanized with carbon dioxide, and necropsy 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 Ps 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 N$_2$, 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 Na$_2$VO$_4$ (10 mM). The homogenate was centrifuged at 14,000 $\times$ 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. Chemiluminescence was detected using 100 μCi [35S]-labeled 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 6.25 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.

RESULTS

The high dose of flutamide decreased the incidence and increased the latency of prostate cancer. Palpable 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 a tumor.
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). Tumor mass of fused ducts was visible as early as 15 weeks (Fig. 2C) in the placebo-treated group, whereas the ducts remained distinct and clear in the flutamide-treated group, as seen at 15 and 20 weeks (Fig. 2, G and 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 5a-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 demonstra-
ble 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 prema-
lignant lesions (5). Thus, a major difference between the two studies
is the timing of androgen deprivation, i.e., early androgen deprivation
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-indepen-
dent 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 possi-
bility 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

![Figure 4](image1.png)

**Fig. 4.** Histology of tumors from the placebo- and flutamide-treated transgenic mice. Representative H&E-stained tissue sections from the normal murine prostate (A; magnification, X66) and various tumors in treated transgenic mice are shown. Both placebo-treated tumors (B; magnification, X66) and low-dose flutamide-treated tumors (C; magnification, X132) were composed of poorly differentiated sheets of malignant cells with no recognizable original glandular prostate architecture. In contrast, the high-dose flutamide-treated tumors (D; magnification, X132) were more differentiated and retained more of the original glandular architecture.

![Figure 5](image2.png)

**Fig. 5.** Effect of flutamide treatment on Tag expression in the TRAMP mouse prostate. Representative Western blot on prostate tissue lysates (40 μg protein/lane) of 10-week-old placebo-treated or flutamide-treated mice. A, top, membrane probed with anti-large Tag mouse monoclonal IgG; bottom panel, membrane reprobed with anti-actin mouse mono-
clonal IgG as internal control. B, densitometric volume of the Tag and actin bands.
mg/day was totally ineffective in the delay or prevention of prostate cancer in the TRAMP mice, we postulate that a threshold level androgen blockade was necessary to elicit its chemopreventive effect. 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 Tag through the androgen-responsive elements of the PB promoter. This, in turn, relieves more of the 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, predisposing 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, because 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 (25). Using the accepted algorithm (26), this translates into 165 mg/day as a chemopreventive dose for treatment in human clinical trials. (26), this translates into 165 mg/day as a chemopreventive dose for treatment.

REFERENCES

Toremifene prevents Prostate Cancer in the TRAMP transgenic model

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ABSTRACT

INTRODUCTION AND OBJECTIVES: The high incidence of prostate cancer and its associated mortality make it imperative to develop prevention strategies. Since both testosterone and estrogens play a role in prostate carcinogenesis, antiandrogens and antiestrogens should have chemopreventive activity against this disease. Unfortunately, antiandrogens have an unfavorable side effects profile in men. Consequently, the possibility that toremifene, an antiestrogen, may have chemopreventive activity was tested in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer.

METHODS: Three groups of mice received subcutaneous implantation of slow release toremifene pellets: (a) low dose toremifene group (6.6 mg/kg/day); (b) high dose toremifene group (33 mg/kg/day); and (c) control placebo group. Efficacy was measured by the absence of palpable tumor. Based on these studies, TRAMP mice were treated with placebo, flutamide (33mg/kg/day), or toremifene (10mg/kg/day). At each time point, 5-10 animals were sacrificed for each treatment group (10, 15, 20, 25 and 30 weeks). Prostate and seminal vesicles were harvested for evaluation by histological, wholmount and molecular techniques. Blood from 5 animals each, was pooled to assay hormone levels.

RESULTS: Both, the high and the low dose toremifene had chemopreventive activity against prostate cancer. Tumor formation was noted in the placebo group (n=10) at week 17, high dose toremifene (n=12) at week 21, and low dose toremifene (n=12) at week 29. This represents an increased latency of up to 12 weeks. By 34 weeks all placebo animals had tumors or died whereas 65% of the toremifene-treated animals were still tumor-free. Although tumor incidence was decreased by both flutamide and toremifene treatment compared to placebo, toremifene was more effective than flutamide. Prostatic intraepithelial neoplasia (PIN) was observed in the prostate tissues of placebo treated but not present in toremifene treated animals. Animal treated with
toremifene had longer survival than placebo treated animals. While serum estradiol levels remained relatively unchanged, both total and free testosterone levels were at higher levels in the toremifene treated group. T antigen (Tag) levels in the prostate tissues of toremifene treated animals at 10 and 15 weeks age were higher than in the comparable placebo-treated group.

CONCLUSIONS: Toremifene is a potent inhibitor of PIN and subsequently leads to significantly decreased in prostate cancer incidence and increase in animal survival. Since treatment with toremifene in the TRAMP does not result in Tag suppression, its mechanism appears to be by local tissue inhibition of androgen action.
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). Prostate cancer is the result of androgen stimulation in the presence of estrogen, and aging (2). In fact, prostate cancer incidence rises more sharply with age than any other malignancy. Chemoprevention is the prevention of cancer by intervening with drugs prior to the invasive or malignant stage of carcinogenesis. It should be emphasized that the focus of chemoprevention is not on the treatment of disease—cancer, but rather it is on the process—carcinogenesis (3). Current studies have established PIN as the precursor of prostatic adenocarcinoma and a marker of men who are at high risk of developing prostate cancer (4). The pre-malignant lesion PIN which represents carcinogenesis can be reversed. Androgen deprivation by flutamide, but not finasteride, decreased the prevalence and extent of PIN and induced epithelial atrophy (5).

Both prostate stroma and epithelium have estrogen receptors and estrogens are clearly implicated in the growth of the prostate (6, 7). The classical estrogen receptor (ER) is ERα, which is predominantly localized in the smooth muscle of the prostatic stroma (8). ERβ, however, appears to be the principal ER in the prostate and is localized to the secretory epithelial cells of the prostate (9, 10). With aging, in many animal species, increasing serum estrogens and decreasing serum androgens and 5α-reductase activity lead to stromal hyperproliferation in the prostate (2). Rising estrogens appears to increase the prostate’s sensitivity to androgens by upregulation of the androgen receptor (AR)(11-13).

Estradiol in the presence of androgens has been shown to stimulate carcinoma in situ and adenocarcinoma of the prostate in Noble rats (14-17). Estradiol is also capable of inducing precancerous lesions and prostate cancer in the aging dog (2). Thus, estrogenic stimulation with decreasing androgen levels may contribute to the genesis of prostate dysplasia and prostate cancer (18-20).
Animal models of prostate cancer currently in use are generally unreliable and the role of antiestrogens in prostate carcinogenesis remained inconclusive (21, 22). One exception, however, is the TRAMP (transgenic adenocarcinoma mouse prostate) model (23). All animals that express the transgene eventually develop prostate cancer that mirrors the human prostate cancer progression (24, 25). The recent development of the TRAMP model has allowed for the first time the capability to directly study chemoprevention (24, 26). In the TRAMP model, the Probasin promoter-SV_{40} large T antigen (PB-Tag) transgene is expressed specifically in the epithelial cells of the murine prostate under the control of the probasin promoter. The probasin promoter contains an androgen response element (ARE) and is androgen dependent. As a result, this model has several advantages over currently existing models (24): 1) Mice develop progressive forms of prostatic epithelial hyperplasia and PIN as early as 10 weeks and invasive adenocarcinoma around 18 weeks of age; 2) Metastatic spread of prostate cancer pattern mimics that of human prostate cancer with common sites of metastases being lymph node, lung, kidney, adrenal gland and bone; 3) Development as well as the progression of prostate cancer can be followed within a relatively short period of 10-30 weeks; 4) Spontaneous prostate tumors arise with 100% frequency; and 5) Animals may be screened for the presence of the prostate cancer transgene prior to the onset of clinical prostate cancer to directly test treatment with chemopreventive agents that may alter prostate carcinogenesis. Using the TRAMP model (23) we reported, earlier, the efficacy of the antiandrogen flutamide in the prevention of prostate cancer (27). In this study, we report that toremifene, a selective estrogen response modifier (SERM), is a potent suppressor of carcinogenesis because it has the ability to reduce PIN, reduce prostate cancer incidence, and increase survival in the TRAMP model.

**MATERIALS AND METHODS**

A study was undertaken to test the efficacy of toremifene in the TRAMP transgenic animal model using the methods described earlier for flutamide (27). The animal experimental protocol was approved by an Institutional Animal Experimentation Review Board and followed the National
Institute of Health guidelines for proper and humane use of animals. The TRAMP (C57BL/6 PB-Tag) transgenic mice were crossbred with FVB wild-type strain, the hybrid litters were screened by PCR for presence of the PB-Tag transgene and only the males that screened positive were used in the study. Toremifene powder was made into slow release pellets (Innovative Research of America, Sarasota, FL) and the drug dose was adjusted for growth related changes in weight. The pellets were implanted subcutaneously through a 1cm incision on the flank into PB-Tag mice (30 days of age, average weight 14 g) anesthetized with metofane (Mallinckrodt, Mundelein, IL).

For palpable tumor study, three groups of 10-12 animals each received a 90 day-release drug pellet of either a low dose toremifene (6.6 mg/kg/day), or a high dose toremifene (33 mg/kg/d) or a placebo pellet. Each treated animal received supplemental dosages at 90 days intervals until tumors were palpable. The efficacy of the treatment was measured by the absence of a palpable tumor. Starting at age 10 weeks animals were evaluated weekly for the presence of a palpable tumor, the endpoint of the study. The statistical analysis compared the differences between treatment groups by Fisher exact test and Wilcoxon’s rank test. All p values were two-sided.

For the effects of toremifene on PIN/tumor incidence study, three cohorts of animals were treated with either placebo or flutamide (33 mg/kg/d) or toremifene (10mg/kg/d) pellets at 4 weeks age. Animals (5-10) from each group were sacrificed at 10, 15, 20, 25 and 30 weeks of age. Blood was pooled and serum stored at -20° C for assay of hormone levels. Serum testosterone and estradiol levels were assayed using the enzyme immunoassay (EIA) kits (DSL-10-4000ACTIVE™) and (DSL-10-4300ACTIVE™), respectively, supplied by Diagnostic Systems Laboratories, Inc. Houston, TX. Values for the sample analyte were determined by interpolation using standards available with the kit.

Wholemounts and Histology: Wholemounts of ventral prostates (7, 10, 15 and 20 and 30 weeks age) and seminal vesicles (7, 10, 15 and 20 weeks age), excised from representative animals in the placebo-treated and high toremifene-treated groups, were examined under dark-field dissection microscope (Olympus SZH stereo- fitted with an Olympus camera). Murine prostate
tissues were harvested, fixed overnight in 4% paraformaldehyde, and processed in Shandon-Lipshaw tissue processor and paraffin-embedded. Tissue sections (4μM thick) were stained with hematoxylin and eosin (H&E) for histological evaluation.

**Western Blot analyses.** Ten crossbred Tag-positive male pups (5 per group) were treated with placebo or flutamide (33mg/kg/d) or toremifene (10mg/kg/d) pellets at 4 weeks-age. Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 and 15 weeks-age, snap-frozen in liquid N₂ and stored at -80°C. Tissue lysates were prepared as previously described (27) and stored at -80°C until used. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Western blot analysis was performed as described earlier (27). Briefly, tissue lysates were loaded onto 7.5% polyacrylamide gels, proteins (40μg/lane) separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (0.2 μm) using a transfer buffer (192 mM glycine, 25 mM Tris-Hcl and 20% methanol). TRAMP prostate tumor tissue was used as positive control. Chemiluminescent Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA) were used as MW standards. Blots were blocked overnight at 4°C in BLOTTO and incubated with the large Tag primary antibody (Pab 101 mouse monoclonal, 1:200, Santa Cruz Biotechnology) for 2 hours at room temperature. The blots were washed (3x) with TTBS (0.05% Tween 20, 50mM Tris-Hcl, 200mM NaCl) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1 hour at 25°C. Androgen receptor expression in placebo or toremifene-treated TRAMP mice (15 and 20 weeks age) was analysed by Western blot using SC# 816 (1:5000), Santa Cruz Biotechnology as primary antibody and (HRP)-conjugated secondary antibody (1:5000). Immunoreactive proteins were visualized on autoradiography film using the enhanced chemiluminescence (ECL) system (APB, Piscataway, NJ). Actin protein expression was used to normalize Tag results. For this purpose, the above membrane was submerged in stripping buffer (100mM 2-mercaptoethanol, 2%SDS, 62.5mM Tris-Hcl pH 6.7) and incubated at 50° C for 30 minutes with occasional agitation. After blocking the membrane was reprobed with actin
primary antibody (1:2500, Chemicon, Temecula, CA) followed by (HRP)-conjugated secondary antibody (1:10000).

RESULTS

Toremifene suppressed palpable tumors in TRAMP mice

Both doses of toremifene were efficacious in the suppression of prostate carcinogenesis in the TRAMP model. Tumors were first palpable by week 17 in the placebo group (n=10), by week 21 in the high dose toremifene (n=12), and by week 29 in the low dose toremifene group (n=12), (Fig. 1). Hence, toremifene increased the latency time of prostate cancer by up to 12 weeks. By 34 weeks, 65% of the toremifene treated animals were still tumor-free, whereas all placebo treated animals had tumors. As the toremifene treated animals never reached the 50% tumor-free point, the time in which 25% of the animals had tumors was instead compared among groups. Tumors were palpable in 25% of the animals by week 24 in the placebo group and by 33-34 weeks in the high and low toremifene treated group. Differences in the presence of palpable tumors between low and high dose toremifene versus placebo treated groups were significant by both Log Rank and Wilcoxon' statistical analysis with a P values < 0.0003 and < 0.00017 for low dose toremifene and high dose toremifene, respectively. These data demonstrated that both high and low dose toremifene significantly decreased the incidence of prostate cancer, increased the latency period, and prolonged the survival of these treated TRAMP mice.

Toremifene prevents formation of prostate cancer as determined by histology and wholemount analyses

The cancer preventive effects of toremifene, using a palpable tumor as the end-point, was further substantiated by histologic and wholemount analysis of prostate tissues harvested from groups of placebo and toremifene-treated animals (10mg/kg/d) at 7, 10, 15, 20, 25, 30, and 33 weeks of age. Wholemount analysis of ventral prostates (Fig. 2) revealed that tumor, as evidenced by fused ducts, was present as early as 15 weeks (Fig. 2, C) in the placebo group. In the toremifene-treated group,
however, the ducts remained distinct and delicate without visible tumor even up to 30 weeks age (Figs. 2, I - J). Before tumor appearance, i.e. at 7 and 10 weeks age, there was no size difference between the placebo and toremifene-treated prostate (Figs. 2A and 2B vs. Figs. 2F and 2G).

Interestingly, TRAMP animals treated with toremifene had suppression of seminal vesicle development compared to placebo-treated control animals (Fig. 3).

Histologically, PIN was seen in the ventral prostate sections of the 7 and 15 week-old placebo-treated animals (Figs. 4, A-B), but not in sections of comparable age toremifene-treated animals (Figs. 4, C-D). In fact, the ducts were clear and resembled that of the 17 week-old wild-type prostate (Fig 5A). The wild type murine prostate had delicate epithelial ducts with very little intervening stroma. In contrast, prostate sections from placebo control TRAMP mice by 17 weeks (Fig. 5B) had complete replacement of the normal prostate ductal architecture by sheets of poorly differentiated, anaplastic cells with a high mitotic index. Toremifene treatment of TRAMP mice preserved the prostatic epithelial ductal architecture which appeared similar to wild type prostate sections (Figs. 4 C-D).

Table 1 summarizes the data on the effect of placebo, flutamide or toremifene treatment on tumor development in the TRAMP model. The placebo treated mice developed prostate tumors by 15-20 weeks of age similar to the palpable tumor study, whereas the toremifene-treated animals had slower rates of prostate cancer formation (up to 33 weeks). More specifically, placebo treated animals had first evidence of prostate cancer in 50% of animals by 15 weeks of age. In contrast, toremifene treated animals had first evidence of prostate cancer by 20 weeks of age, but only in 14% of animals. By 20 weeks of age, 100% of placebo-treated animals had prostate cancer. The time for 50% of placebo animals to develop tumors was 15 weeks compared to only 43% of the animals had tumors by 33 weeks in the toremifene-treated animals. Interestingly, the tumor incidence was about 50% lower with flutamide treatment (43%, 50% and 57%) and about 75% lower with toremifene treatment (14%, 20% and 28%) at the comparable ages. Thus, toremifene appeared to be a more potent chemopreventive agent than the antiandrogen flutamide. Hence, these
data further confirm that even with a more sensitive assessment of tumorigenicity, toremifene had significant chemopreventive activity.

**Hormonal profile of toremifene treated animals**

Serum free and total testosterone and estradiol levels were measured using the enzyme immunoassay. Toremifene did not affect serum estradiol levels, which remained statistically almost unchanged between 10-30 weeks age. In toremifene-treated animals, serum total testosterone levels were elevated at 10-15 weeks and returned to levels that were comparable to placebo-treated animals by 20-30 weeks. In contrast, serum free testosterone remained elevated from 10 to 30 weeks age compared to placebo treated animals (Table 2). Thus, chronic use of toremifene in male animals resulted in restoration of total testosterone, but free testosterone levels remained elevated.

**Large T-antigen is not down regulated with toremifene treatment in the TRAMP model**

One major concern was that the observed chemopreventive effect of toremifene might be a consequence of direct suppression of the probasin promoter by toremifene resulting in reduced expression of the Large Tag transgene. The probasin promoter has an ARE and if this chemopreventive effect is mediated by blocking androgen dependent pathways, then probasin promoter activity may be inhibited. Consequently, T-antigen expression was determined by Western blot analysis and representative data are shown (Fig. 6). The oncoprotein was clearly present in the prostate tumor tissue resected at 20 weeks age as well as in the placebo-treated prostate at 15 weeks age (Fig. 6, A). Surprisingly however, toremifene-treated prostate Tag level was higher than the placebo-treated prostate. Similar results were obtained with 10 week old toremifene-treated prostate tissue (Fig. 6, B) in which Tag expression maintained at levels higher than the comparable placebo-treated prostate tissues. Thus, the chemopreventive activity of toremifene on TRAMP prostate cancer was not a result of suppression of probasin activity and Large Tag expression.
Androgen receptor is not down regulated with toremifene treatment in the TRAMP model

The hybrid TRAMP (TRAMP x FVB) tumor tissue had much higher level of androgen receptor than the prostate tissues of nontransgenic mouse of the same genetic background (C57/BL6 x FVB) (Fig. 7). Interestingly, the prostatic androgen receptor levels were similar for placebo and toremifene treated TRAMP mice and resembled that from the tumor tissue. Thus toremifene treatment did not significantly affect the androgen receptor expression.

DISCUSSION

SERMs are generally considered as “weak estrogens” because they possess both agonist and antagonist activities that are dependent on the specific tissue type studied and the interaction of a particular agent with ER receptor subtype (28). Several of the animal models of prostatic cancer that are currently in use have shown that tamoxifen, another SERM, is able to suppress prostate tumor formation (21, 22). The TRAMP model is unique, in that each animal that has the transgene eventually develops prostatic cancer that mirrors the human prostatic cancer progression (24, 25). In this study, toremifene demonstrated chemopreventive activity by significantly reducing the incidence of prostate cancer in TRAMP mice compared to the placebo-treated control group. This is the first report on the chemoprevention potential of a SERM in an autochthonous animal model of primary prostate cancer. The significant increase in the latency period of prostate cancer observed in toremifene-treated animals suggests that toremifene is able to suppress carcinogenesis. In contrast with our results on the antiandrogen flutamide (27), inhibition of carcinogenesis by toremifene was not a result of direct suppression of the probasin promoter and reduction in Large T levels.

Tamoxifen has been previously shown to down regulate androgen receptor expression as one of the mechanisms that a SERM could suppress androgen dependent tissues (6, 7, 29, 30). The seminal vesicles, like the prostate, are androgen-dependent. Consistent with this possible mechanism, toremifene inhibited androgen dependent seminal vesicle development in the presence
of elevated serum free testosterone levels suggesting that toremifene appears to be acting as an antiandrogen. However, many of our experimental observations do not support this antiandrogenic mechanism: 1) Toremifene did not suppress the probasin promoter which contains an ARE, 2) The size of the prostate glands were similar for the toremifene and placebo treated animals prior to 15 weeks of age, 3) Prostatic androgen receptor levels were similar for toremifene and placebo treated TRAMP mice, and 4) Prostate cancer formation was inhibited in a milieu of elevated free testosterone levels. Thus, the mechanism of toremifene chemopreventive activity appears to be through nonandrogenic pathways. In fact, toremifene was a more potent chemopreventive agent than antiandrogen flutamide.

Recently, a new ER, ERβ (also known as ERβ1), was cloned from a rat prostatic cDNA library and is present in murine and human prostates (9, 10, 31, 32). Consequently, the previous ER is now designated as ERα. ERα and ERβ share high amino acid homology (DNA binding domain 95% and ligand binding domain 55%), have the same affinity for estradiol, and can hetero- or homodimerize to form a signaling dimeric complex (9, 10). Although estradiol activates both ERα and ERβ, ERα stimulates transcription and cellular proliferation, while ERβ quenches ERα activation (33). Other ER receptors have been also recently cloned from prostate including ERβ2 which has 1000-fold less affinity for estradiol (34, 35) and ERβcx which has no affinity for estradiol (29). All ER subtypes can form heterodimers with each other. Adding to the complexity of the ER receptor-mediated mechanisms of action is the involvement of coregulators that are required for ER signaling. These coregulators include coactivators, corepressors, and integrators (30, 34, 35).

Prostatic stroma and epithelium both express estrogen receptors, and estrogens are clearly implicated in the growth of the prostate (6, 7) In the rodent prostate, ERα is present in the stroma, whereas ERβ is located in the secretory luminal epithelial cells of the prostate (9, 10, 17, 31, 32). ERβ knockout mice develop prostate hyperplasia with aging supporting the contention that ERβ
normally suppresses prostate epithelial proliferation (36) In contrast, ERα, not ERβ, is the predominant ER in the female reproductive system (9, 10). This observation is critical to interpreting published findings because earlier data about ER in the prostate must be reevaluated because past studies were unable to distinguish between ERα and ERβ. SERMs have the ability to bind to ERα and ERβ to compete with estradiol and other estrogens for binding to estrogen receptors in breast and prostate tissue (9, 33, 37-39). Formation of SERM-ER complexes result in the local inactivation of the estrogen regulated genes, thereby, decreasing cellular proliferation. Consequently, toremifene may be exerting its chemopreventive effects through stimulation of ERβ or blocking ERα.

Estrogen stimulates cellular proliferation through ER by inducing local production of stimulatory peptide growth factors including transforming growth factor α (TGFα), insulin-like growth factor (IGF), and epidermal growth factor (EGF) and by inhibiting the local expression of growth inhibitory factors like transforming growth factor β (TGFβ) (40, 41). SERMs, therefore, would be expected to act at the cellular microenvironment level to decrease the amounts of these stimulatory growth factors and augment the production of TGFβ. In addition, the antiproliferative effects of SERMs may be mediated by other intracellular signaling mechanisms including binding and sequestration of calmodulin (42) inhibition of protein kinase C (43, 44) and induction of p21^{waf1/cip1} (44). Nonetheless, the exact mechanism of toremifene chemopreventive activity remains to be elucidated.

In conclusion, toremifene demonstrates chemopreventive activity in the TRAMP model of human prostate cancer. Toremifene appears to increase latency period for tumor formation, decreases of prostate cancer incidence, and increases survival. The mechanism of the chemopreventive effects of toremifene is through nonandrogenic pathways. Toremifene is a SERM that may have potential use in prostate cancer chemoprevention clinical trials.
ACKNOWLEDGEMENTS

We thank Dr. Ronald Couch (Coulston Foundation, White Sands Research Center, Alamogordo, New Mexico) for measuring serum hormone levels.

REFERENCES


27. Raghow, S., Kuliyev, E., Steakley, M., Greenberg, N. and Steiner, M. S. Efficacious


Figure 1. Chemopreventive effects of toremifene in the TRAMP model. Transgenic mice were divided into three groups: (a) placebo; (b) low-dose toremifene (6.6 mg/kg/d) and (c) high-dose toremifene (33 mg/kg/d). 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.

Figure 2. Effect of toremifene treatment on prostate tumor development in the TRAMP model. Dark-field microscopy of ventral prostate whole mounts showing prostatic ducts joining the urethra. A-E, placebo-treated; and, F-J, high-dose toremifene-treated prostate.

Figure 3. Effect of toremifene treatment on seminal vesicle development in the TRAMP model. Darkfield microscopy of seminal vesicle wholemounts from placebo and toremifene treated animals.

Figure 4. Prostate tumor development and histology of tumors in the placebo- and toremifene-treated TRAMP mice. (A-B), 7 and 15 week old placebo-treated prostate showing H&E stain of ventral prostate with PIN and early stage tumor; (C-D), 7 and 15 week old toremifene-treated prostate showing H&E stain of ventral prostate with no PIN (magnification, A & C, 33x; B & D, 13.2x).

Figure 5. Histology of wildtype mouse ventral prostate and TRAMP ventral prostate (tumor) at 17-weeks age. H&E stain, magnification (x 66).

Figure 6. Effect of placebo and toremifene (10mg/kg/d) treatment on Tag expression in the TRAMP mouse prostate. A, Representative Western Blots using prostate tissue lysates (40μg
protein/lane) of 15-week old placebo or toremifene treated mice; top, membrane probed with anti-large Tag mouse monoclonal IgG; bottom panel, membrane reprobed with anti-actin mouse monoclonal IgG as internal control. Prostate tumor from a 20 week-old untreated TRAMP mouse was used as positive control; B, Western blot of prostate tissue lysates from 10 week-old mice. top and bottom panel same as A.

**Figure 7.** Effect of placebo (PL) and toremifene (T) treatment on androgen receptor expression in the TRAMP model. TRAMP animals were treated with toremifene (10mg/kg/d) at 4 weeks age and prostate tissues of 15 and 20 week-old animals were analysed by Western blot. Tissues from comparable age nontransgenic animals (NT) were used as control for the TRAMP; top, membrane probed with anti-AR antibody SC#816, rabbit polyclonal IgG; bottom panel, membrane reprobed with anti-actin mouse monoclonal IgG as internal control.

**Table 1.** Effect of placebo, flutamide or toremifene treatment on incidence of prostate tumor development in the TRAMP model. Three cohorts of animals were treated with either placebo, or flutamide (33 mg/kg) or toremifene (10 mg/kg/d) pellets at 4 weeks age and 5-10 animals from each group were sacrificed at 10, 15, 20, 25 and 30 weeks age to examine for presence of tumor.

**Table 2.** Effect of placebo or toremifene treatment on serum testosterone and estradiol levels. Three cohorts of animals were treated with either placebo or toremifene (10 mg/kg/d) pellets at 4 weeks age. Animals (5-10) from each group were sacrificed at 10, 15, 20, 25 and 30 week age. Blood was pooled to obtain serum and stored at -20° C for assay of hormone levels. Serum testosterone and estradiol levels were assayed using the enzyme immunoassay (EIA) kits (DSL-10-4000ACTIVE™) and (DSL-10-4300ACTIVE™), respectively, supplied by Diagnostic Systems Laboratories, Inc. Houston, TX. Values for the sample analytes were determined by interpolation using standards available with the kit.
Table 1. Effect of placebo, flutamide or toremifene treatment on incidence of prostate tumor development in the TRAMP model. Three cohorts of animals were treated with either placebo, or flutamide (33 mg/kg/d) or toremifene (10 mg/kg/d) pellets at 4 weeks age and 5-10 animals from each group were sacrificed at 10, 15, 20, 25 and 30 weeks age to examine for presence of tumor.

<table>
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<tr>
<th>Treatment</th>
<th>10-wk</th>
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<th>20-wk</th>
<th>25-wk</th>
<th>30-wk</th>
<th>33-wk</th>
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<tr>
<td>Placebo</td>
<td>0%(0/10)</td>
<td>50%(4/8)</td>
<td>100%(5/5)</td>
<td>83%(5/6)</td>
<td>100%(7/7)</td>
<td>all died</td>
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<tr>
<td>Flutamide (33mg/kg)</td>
<td>0%(0/6)</td>
<td>0%(0/10)</td>
<td>43%(3/7)</td>
<td>50%(3/6)</td>
<td>57%(4/7)</td>
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<tr>
<td>Toremifene (10mg/kg)</td>
<td>0%(0/12)</td>
<td>0%(0/9)</td>
<td>14%(1/7)</td>
<td>20%(1/5)</td>
<td>28%(2/7)</td>
<td>43%(3/7)</td>
</tr>
</tbody>
</table>

*=percent of animals with tumor; ( ), actual number of animals, * discontinued.

Table 2. Effect of placebo or toremifene treatment on serum testosterone and estradiol levels. Three cohorts of animals were treated with either placebo or toremifene (10 mg/kg/d) pellets at 4 weeks age. Animals (5-10) from each group were sacrificed at 10, 15, 20, 25 and 30 week age. Blood was pooled to obtain serum and stored at -20° C for assay of hormone levels. Serum testosterone and estradiol levels were assayed using the enzyme immunoassay (EIA) kits (DSL-10-4000ACTIVE™) and (DSL-10-4300ACTIVE™), respectively, supplied by Diagnostic Systems Laboratories, Inc. Houston, TX. Values for the sample analytes were determined by interpolation using standards available with the kit.

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<th>20-wk</th>
<th>25-wk</th>
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<td>Total testosterone (ng/ml)</td>
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<td>Placebo</td>
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<td>Placebo</td>
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<td>36.89</td>
<td>48.10</td>
<td>36.89</td>
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*= no sample.
Fig. 1.

[Graph showing the percentage of tumor-free animals over weeks for Placebo and two different doses of treatment: High (33 mg/kg/d) and Low (6.6 mg/kg/d). The graph indicates the number of animals (n) for each treatment group: Placebo n=10, High n=12, and Low n=12.]
Fig. 2

7 wk  10 wk  15 wk  20 wk  30 wk

Placebo

A  B  C  D  E

Toremifene

F  G  H  I  J
A-Wild-type mouse ventral prostate-17wk

B-TRAMP ventral prostate-17wk
Western Blot.

Fig. 6

(A) Western Blot

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<tr>
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<th>Toremifene</th>
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<td>90kD</td>
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</tr>
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<td>43kD</td>
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(B) Western Blot

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<td>132kD</td>
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<td></td>
<td>T-antigen (94kD)</td>
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<tr>
<td>90kD</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>62kD</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Actin (45kD)</td>
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</table>

Fig. 7

Western Blot

<table>
<thead>
<tr>
<th>TRAMP Tumor</th>
<th>NT-15 wk</th>
<th>PL-15 wk</th>
<th>T-15 wk</th>
<th>NT-20wk</th>
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AR (104 kD) | Actin (43 kD)