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Nursinha Camara
09/30/2002
Role of the TGFβ1 in the Prevention of Prostate Cancer

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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 was tested through two specific aims: 1) whether the chemopreventive effects of antiandrogens, antiestrogens and retinoic acid are mediated by TGFβ, 2) whether prostate cancer may be prevented in the TRAMP model at the genetic level by crossing with transgenic mice engineered to overexpress TGFβ. The retinoid MDI301 was ineffective. Both flutamide and toremifene delayed onset of prostate cancer but by different mechanisms: flutamide inhibited but toremifene did not affect large T-antigen expression. Toremifene treatment elevated total and free testosterone levels but did not affect androgen receptor levels suggesting its action through non-androgenic pathway. Toremifene treatment elevated total and free testosterone levels but did not affect androgen receptor levels suggesting its action through non-androgenic pathway. We explored if toremifene chemopreventive activity involved TGFβ pathway. Toremifene had no influence on TGFβ1 and TGFβ2 but elevated TGFβ3. Since both TGFβRI and RI were absent in the TRAMP prostate and toremifene failed to restore their expression, it appears that toremifene action is independent of the TGFβ pathway. The results of crossbreeding experiments showed that overexpressed TGFβ in prostate or seminal vesicles delayed tumor development in 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 – vimentin 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, 2000,13 (reprint attached) and "Toremifene Prevents Prostate Cancer in the Transgenic Adenocarcinoma of Mouse Prostate Model" by Raghow et al. Cancer Res. 62: 1370-1376, 14 (reprint 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).14 The chemopreventive delay of prostatic tumors by flutamide and toremifene beyond 20 weeks was also quite apparent.13,14 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).14 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 SV40 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 N₂ 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.¹³,¹⁴

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).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)¹³ was replaced by sheets of undifferentiated, anaplastic cells with a high mitotic index (Fig. 4, B).¹³ Tumors from the low dose flutamide-treated (Fig. 4, C)¹³ group were similar to those of the placebo group. In contrast, the high dose flutamide-treated (Fig. 4, D)¹³ mice had tumors that were distinctively differentiated retaining a glandular architecture; the mitotic index was much lower than the placebo group.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 SV₄₀ 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 SV₄₀ T-antigen expression driven by the probasin promoter. No experiment is proposed to address the effects of antiandrogens, antiestrogens and retinoic acids on the SV₄₀ 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).¹³ 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.¹³

The efficacy of toremifene was significantly higher than of the comparable flutamide doses (Table 1).¹⁴ 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)¹⁴ but not in the prostate tissues of comparable 7 and 15 week-old toremifene-treated animals (Fig. 4, C-D).¹⁴ 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. T-antigen expression was determined by WB 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 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.\textsuperscript{3-6} 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.\textsuperscript{19} 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.\textsuperscript{20} 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.\textsuperscript{21} 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\textsuperscript{22,23}.

Changes in TGFβ related to flutamide or toremifene-treatment were investigated. Prostates of 7, 10 and 15 week-old age-matched non-transgenic mice were used as controls for the TRAMP mice. Our data showed that compared with the prostates of age-matched non-transgenic mice, TGFβ1 (Fig. 2, A) and TGFβ3 (Fig. 2, C), but not TGFβ2 (Fig. 2, B) are down-regulated in the TRAMP mice. Treatment with flutamide did not affect TGFβ1 expression which remained absent at all ages that were tested, but TGFβ2 and TGFβ3 mRNA levels were higher at 10 and 25 weeks in mice treated with flutamide. More importantly, while toremifene did not affect TGFβ1 and down-regulated TGFβ2 expression after 15 weeks of age, it elevated levels of TGFβ3 at 7, 10 and 15 weeks age.

TGFβRI (Fig. 3, A) and TGFβRII (Fig. 3, B) mRNAs were absent in the prostates of 7 week-old non-transgenic mice and only low levels of these receptors were present at 10 and 15 weeks of age. In the prostates of TRAMP mice, RT-PCR detected no TGFβRI (Fig. 3, A) or TGFβRII (Fig. 3, B) mRNA. Moreover, neither flutamide nor toremifene-treatment could restore TGFβRI (Fig. 3, A) or TGFβRII (Fig. 3, B) expression in the TRAMP prostate. Thus, there is loss of both TGFβRI and TGFβRII during prostate carcinogenesis in the TRAMP mice.

Taken together, the data on TGFβ growth factors and receptors in TRAMP mice suggest: 1) loss of TGFβ1 and TGFβ3 is associated with prostate carcinogenesis, 2) loss of both receptors TGFβ-R1 and TGFβ-R2 accompanies prostate carcinogenesis in TRAMP mice and, 3) toremifene treatment of TRAMP mice increased expression of prostatic TGFβ3 compared to the placebo-treated mice. 4) increase in TGFβ3 levels by toremifene is inconsequential to prostate cancer prevention because TGFβ signal pathway remains disrupted due to absence of TGFβ-R1 and TGFβ-R2 receptors in the TRAMP prostate. Thus, the chemopreventive activity of toremifene appears to be independent of the TGFβ pathway.

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 was used with some modification (reprint attached). 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). However, IP on drug treated samples was not feasible due to insufficient amount of prostate tissue. TGFβ protein was detectable only in MMTV-TGFβ seminal vesicles (Fig. 4C) by WB. Concurrent with the low mRNA levels seen in general, TGFβ growth factor or receptor proteins were absent or below the detection limits of immunohistochemical technique used.

Additionally, ERα mRNA was significantly lower 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 wholemount 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). Two manuscripts “Raghow S and Steiner M. Prostate-targeted overexpression of TGFβ in a transgenic mouse model” and “Raghow, S. and Steiner, M. Suppression of prostate cancer in the TRAMP mice by toremifene is independent of TGFβ signal pathway” are 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 wholemount 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. By 25 and 30 weeks age, 29% and 50% of the TRAMP x PB-TGFβ animals had tumors.
In comparison, over 60% of TRAMP x MMTV-TGFβ animals had tumors at 25 and 30 weeks age. 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. The autocrine influence of TGFβ in inhibition of prostate cancer was longer relative to the paracrine pathway.

Samples were collected for histological and molecular studies for 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 enabled us to better focus on the TGFβ signaling pathway intermediates such as p21. TGFβ protein was detectable only in MMTV-TGFβ seminal vesicles by WB. TGFβ growth factor or receptor proteins were absent or below the detection limits of immunohistochemical technique used.

List Of Personnel

Dr. Mitchell Steiner: Principal Investigator, 1998-2002
Dr. Sharan Raghow: Co-Investigator, 1998-2002
Dr. Massoumeh Z Hooshdaran: Post-doctoral fellow, 2000-2002
Dr. Sanjay Katiyar: Post-doctoral fellow, 1999-2001
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 (MDI-301) did not exhibit chemopreventive activity against prostate cancer in the TRAMP.

- 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

- Toremifene inhibited prostate cancer in a milieu of elevated free testosterone levels and toremifene treatment did not alter prostatic androgen receptor 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

- TGFβ overexpression in the prostate (PB-TGFβ) or seminal vesicles (MMTV-TGFβ) delayed tumor development in the TRAMP mice only up to 15 and 20 weeks age, respectively

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

- The loss of TGFβ1 and TGFβ3, but not TGFβ2, is associated with TRAMP prostate carcinogenesis

- The loss of both receptors, TGFβ-RI and TGFβ-RII, occurs during TRAMP prostate carcinogenesis. Neither flutamide, nor toremifene treatment was able to restore expression of TGFβ receptors.

- Toremifene treatment of TRAMP mice had no effect on TGFβ1, but increased expression of TGFβ3 compared to the placebo-treated mice. This increase in TGFβ3 is inconsequential to prostate cancer prevention as TGFβ signal pathway remains disrupted due to absence of TGFβ-RI and TGFβ-RII receptor expression in the TRAMP prostate. Thus, the chemopreventive activity of toremifene is independent of the TGFβ pathway.

REPORTABLE OUTCOMES

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

2. **Abstracts:**
3. Manuscripts:
- Raghow, S. and Steiner, M. Prostate-targeted expression of TGFβ in a transgenic mouse model (in preparation).
- Raghow, S. and Steiner, M. Suppression of prostate cancer in the TRAMP mice by toremifene is independent of TGFβ signal pathway (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. Although toremifene treatment stimulated TGF3 mRNA production, it was unable to restore expression of the key receptors TGFβ-RI and TGFβ-RII and consequently the disrupted TGFβ signal pathway. Thus, the toremifene chemopreventive activity appears to be independent of TGFβ pathway. We speculate that toremifene chemopreventive activity may involve estrogen signal pathway. Our work showed that 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 cRA MDI 301 vs. the Placebo on Seminal Vesicle Development in the TRAMP mouse.
Fig. 2A. RT-PCR. Effect of flutamide and toremifene on TGFβ1 expression in the TRAMP mouse prostate at 7, 10, 15, 20 and 25 weeks of age. Prostate tissue from age matched non-transgenic mouse was used as control for the TRAMP mice.
Fig. 2B. RT-PCR. Effect of flutamide and toremifene on TGFβ 2 expression in the TRAMP mouse prostate at 7, 10, 15, 20 and 25 weeks of age. Prostate tissue from age matched non-transgenic mouse was used as control for the TRAMP mice.
Fig. 2C. RT-PCR. Effect of flutamide and toremifene on TGFβ 3 expression in the TRAMP mouse prostate at 7, 10, 15, 20 and 25 weeks of age. Prostate tissue from age matched non-transgenic mouse was used as control for the TRAMP mice.

<table>
<thead>
<tr>
<th>Non-transgenic mouse (NT)</th>
<th>TRAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marker</strong></td>
<td><strong>Placebo</strong></td>
</tr>
<tr>
<td>7 wk</td>
<td>10 wk</td>
</tr>
<tr>
<td>7 wk</td>
<td>10 wk</td>
</tr>
<tr>
<td>7 wk</td>
<td>10 wk</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>(415 bp)</td>
</tr>
<tr>
<td>Actin</td>
<td>(470 bp)</td>
</tr>
</tbody>
</table>
Fig. 3A. RT-PCR. Effect of flutamide and toremifene on TGFβ-RI expression in the TRAMP mouse prostate at 7, 10, 15, 20 and 25 weeks of age. Prostate tissue from age matched non-transgenic mouse was used as control for the TRAMP mice.

<table>
<thead>
<tr>
<th>Non-transgenic mouse (NT)</th>
<th>TRAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marker</strong></td>
<td><strong>Placebo</strong></td>
</tr>
<tr>
<td>7 wk</td>
<td>7 wk</td>
</tr>
<tr>
<td>10 wk</td>
<td>10 wk</td>
</tr>
<tr>
<td>15 wk</td>
<td>15 wk</td>
</tr>
<tr>
<td>20 wk</td>
<td>20 wk</td>
</tr>
<tr>
<td>25 wk</td>
<td>25 wk</td>
</tr>
<tr>
<td>+ve</td>
<td></td>
</tr>
</tbody>
</table>

**TGFβRI**
(986 bp)

**Actin**
(470 bp)
Fig. 3B. RT-PCR. Effect of flutamide and toremifene on TGFβ-RII expression in the TRAMP mouse prostate at 7, 10, 15, 20 and 25 weeks of age. Prostate tissue from age matched non-transgenic mouse was used as control for the TRAMP mice.
Fig. 4 A. p21 Western Blot

C-32 protein extract (+ve control (200g)  PB-VP  PB-CG  PB-SV  MMTV-VP  MMTV-CG  MMTV-SV  Chem. Marker

P21  

23 KD

Fig. 4 B. p21 Immunoprecipitation

PB-VP  PB-CG  PB-SV  MMTV-VP  MMTV-CG  MMTV-SV  Chem. Marker

P21  

23 KD
Fig. 4 C. TGFβ Western Blot
Fig. 5. RT-PCR analyses showing differential TGFβ expression in tissues of transgene-positive pups A630, A631, A634 and A650 from Founder PBTGFB 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 T 2371. 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, MMTV TGFβ 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, MMTVTGFβ 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. %=percent of animals with tumor; ( ), actual number of animals.

<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>a</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>

a, 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) 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>
<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</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Placebo</td>
<td>0.24</td>
<td>0.09</td>
<td>0.27</td>
<td>0.07</td>
<td>0.13</td>
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<tr>
<td>Toremifene</td>
<td>5.41</td>
<td>7.80</td>
<td>0.12</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Free testosterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.59</td>
<td>0.88</td>
<td>0.98</td>
<td>0.50</td>
<td>0.21</td>
</tr>
<tr>
<td>Toremifene</td>
<td>28.22</td>
<td>13.65</td>
<td>31.94</td>
<td>3.78</td>
<td>9.63</td>
</tr>
<tr>
<td><strong>Estradiol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>37.10</td>
<td>17.73</td>
<td>23.78</td>
<td>38.29</td>
<td>30.22</td>
</tr>
<tr>
<td>Toremifene</td>
<td>39.51</td>
<td>36.89</td>
<td>48.10</td>
<td>36.89</td>
<td>a</td>
</tr>
</tbody>
</table>

a, 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>
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<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>No SV, AP development</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>
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<td></td>
<td></td>
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<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>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>AP, RNA degraded??</td>
<td></td>
</tr>
<tr>
<td>T2371-M</td>
<td>A634-M</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OV, UT, BR</td>
</tr>
<tr>
<td>T2371-M</td>
<td>A642-F</td>
<td>ovary uterus+ breast+</td>
<td>+</td>
<td>0</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 20-F</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</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 ≤ 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>

<table>
<thead>
<tr>
<th>Crossbred strain</th>
<th>Tumor (+)</th>
<th>Tumor (-)</th>
<th>Total</th>
<th>% tumor</th>
<th># died (no tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (25 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAMP x FVB</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>83%</td>
<td>3</td>
</tr>
<tr>
<td>TRAMP x PB-TGFβ</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>29%</td>
<td></td>
</tr>
<tr>
<td>TRAMP x MMTV-TGFβ</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>66%</td>
<td>2</td>
</tr>
<tr>
<td>Age (30 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAMP x FVB</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>100%</td>
<td>3</td>
</tr>
<tr>
<td>TRAMP x PB-TGFβ</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>TRAMP x MMTV-TGFβ</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>60%</td>
<td></td>
</tr>
</tbody>
</table>

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, 15.5, 18 and 20 weeks in gestation were 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 antirat 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 antiporcine 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 primary antibody) controls. After a thorough cold phosphate buffered saline rinse (5 minutes × 3 with agitation on a platform shaker), the samples were incubated for 1 hour with the appropriate biotinylated secondary antibody (1:1,000 in 0.5% casein/phosphate buffered saline, rabbit antigoat IgG for TGF-β1 and TGF-β3, and goat antirabbit for TGF-α and TGF-β2). The signal was further enhanced by a 30-minute incubation in streptavidin-horseradish peroxidase tracer complex that recognizes the biotin labeled secondary antibody. Immunodetection on sections rinsed in cold phosphate buffered saline used 3-amino, 9 ethyl-carbazole chromogen and hydrogen peroxide as substrate for 5 minutes. After a light counterstain (2.5 minutes) with Mayer's hematoxylin and a thorough rinse of the sections with tap water, coverslips were placed using an aqueous based mounting medium.

Light microscopic analysis of the epithelial and stromal compartments of prostatic tissue were scored for immunoreaction color intensity (reddish brown) of the peroxidase product by 2 independent investigators. The estimated visual intensity was rated from no staining to intense staining. For each fetal age group both independent scores of all samples representing different regions of the prostate were assessed and assigned an intensity value (see table).

RESULTS

Figure 1 represents negative and positive controls showing the immunostaining specificity of TGF-α and TGF-β primary antibodies. Localization of peptide growth factor immunostaining revealed that by 9.5 weeks of gestation TGF-α, TGF-β1 and TGF-β3 were present in the mesenchyme (fig. 2, A, B and D); whereas staining for mesenchymal TGF-β2 was negligible (fig. 2, C). Epithelial staining for all growth factors studied was either absent or present at low levels during this early period of prostatic growth (fig. 2).

Localization of 5α-reductase between 13 and 16.5 weeks of gestation persisted primarily in the mesenchyme, showing little change in response to the androgen surge at 13 weeks. However, epithelial androgen receptor staining becomes more intense with higher androgen levels (unpublished data). DHT produced in the stroma influenced the epithelium by paracrine pathway 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, C and D). 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 prostatic morphogenesis.

DISCUSSION

Growth factors have been implicated in benign and malignant growth as possible autocrine and paracrine mediators of...
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 5α-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 recognized as male pseudohermaphroditism 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. 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-β families. TGF-α, TGF-β1 and TGF-β3 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-β2 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-α, TGF-β1 and TGF-β3. 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 greatest intensity of immunostaining for TGF-α and TGF-β3 in the epithelium during 13 to 16.5 weeks of gestation. Although TGF-α, TGF-β1 and TGF-β3 were present initially in the mesenchyme, they later appeared in the epithelium during the androgen surge. Hence, it appears that initially DHT only induced or influenced 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-β3 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 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 prostatic 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-β1, 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

SELECTIVE ESTROGEN RECEPTOR MODULATORS FOR THE CHEMOPREVENTION OF PROSTATE CANCER

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ABSTRACT

The ability to interfere with prostate carcinogenesis, and as a consequence, prevent prostate cancer with drugs is the basis for chemoprevention. The prostate contains estrogen receptors in both the stroma and epithelium. Both animal models and human epidemiologic studies have implicated estrogens as an initiator of prostate cancer. In the aging male, prostate cancer occurs in an environment of rising estrogen and decreasing androgen levels. Selective estrogen receptor modulators (SERMs) have shown the ability to prevent (GTx-006 [acapodene]) and treat (GTx-006 and arzoxifene) prostate cancer, suggesting that they may be used in prostate cancer chemoprevention. A phase 2 clinical trial using GTx-006 for prostate cancer chemoprevention is currently being conducted.

Prostate cancer is 1 of the most frequent cancers among men in the United States, with more than 184,500 new diagnoses expected in 2000. Unfortunately, more than 60% of these newly diagnosed cases of prostate cancer will eventually be found to be pathologically advanced; in such cases, there is no cure and the prognosis is dismal. One approach may be to find cancer earlier through screening programs, thus reducing the number of advanced prostate cancer patients. Another strategy is chemoprevention—the prevention of cancer by intervening with drugs before the invasive or malignant stages of prostatic carcinogenesis. It should be emphasized that the focus of chemoprevention is not treatment of disease (cancer) but rather the disease process (carcinogenesis). New innovative approaches are urgently needed at both the basic science and clinical levels. The development of effective chemopreventive strategies against prostate cancer should have significant medical and economic impact on treatment and outcome in this area of greatly unmet medical need.

EVIDENCE THAT ESTROGENS ARE CRITICAL TO PROSTATIC GROWTH—THE ESTROGEN RECEPTORS STORY: A GROWING FAMILY OF RECEPTORS

Estrogens play an important role in reproductive, central nervous, skeletal, and cardiovascular systems of both males and females. The estrogen receptor (ER) is the only member of the steroid subfamily of nuclear receptors that has different subtypes. Recently, a new ER, ERβ (also known as ERb1), was cloned from a rat prostatic cDNA library and is present in murine and human prostates. 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 heterodimerize or homodimerize to form a signaling dimeric complex. Although estradiol activates both ERα and ERβ, ERα stimulates transcription and cellular proliferation, while ERβ quenches ERα activation. Interestingly, ERβ also stimulates production of quinone reductase and glutathione S-transferase, which are chemoprotective detoxification enzymes. Other ER receptors have been also recently cloned from prostate, including ERβ2 (1,000-fold less affinity for estradiol) and ERβcx (no affinity for estradiol). 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.13,14,16

Prostatic stroma and epithelium both express estrogen receptors, and estrogens are clearly implicated in the growth of the prostate.17,18 In the rodent prostate, ERα is present in the stroma, whereas ERβ is located in the secretory luminal epithelial cells.6–9,19 ERβ knockout mice develop prostate hyperplasia with aging, supporting the contention that ERβ normally suppresses prostate epithelial proliferation.20 In contrast, ERα, not ERβ, is the predominant ER in the female reproductive system.6,7 This observation is critical to interpreting published findings, because earlier data about ER in the prostate must be reevaluated as past studies were unable to distinguish between ERα and ERβ.

Although ERβ has been detected in normal and malignant human prostate and prostate cancer cell lines, ERβ may not be the predominant ER subtype expressed in vivo.13,19,21–23 In normal prostate and BPH, ERα is localized in the stromal cells and estrogens mediate their effects on the prostatic epithelium through paracrine pathways.24–28 In prostatic cancer, however, Bonkhoff et al.29 have demonstrated the presence of ERα, not ERβ, in premalignant and malignant prostatic epithelium. ERα was present in 11% of high-grade prostatic intraepithelial neoplasia (HGPIN) and 61% of prostate cancer cases.29 Bodker et al.30 demonstrated ERα in 6 of 11 prostate cancer cases. Nonetheless, ERβ1 and ERβ2 have also been detected in human prostate and prostate cancer cell lines by RNA nuclease protection assays and reverse transcription polymerase chain reaction. The exact contribution of these other ER subtypes to normal and malignant prostate cell biology, however, remains to be elucidated.13,19,21–23,31

**Clinical and Experimental Evidence**

Increasing serum estrogens, decreasing serum androgens, and 5α-reductase activity have been demonstrated to lead to age-dependent stromal hyperproliferation in many animal species.32 Rising estrogens appear to increase the prostate's sensitivity to androgens by upregulation of the androgen receptor (AR).31,32,34 Estrogens can induce benign prostatic hypertrophy (BPH) in humans and dogs only in the presence of androgens.35,36 Interestingly, estrogens appear to “imprint” the developing neonatal murine prostate's ability to become dysplastic and develop tumors with aging.18,37–39 Recently, Shibata et al.40 evaluated the transition zone of the aging human prostate and found a time-dependent decrease in dihydrotestosterone (DHT) with a concomitant increase in the estradiol/DHT ratio. This increased estradiol/DHT ratio has been proposed to be responsible for a relatively estrogen-dominant environment in aging males. Moreover, African Americans have the highest levels of serum estrone and estradiol, whereas Japanese men have the lowest; this parallels their respective risks for development of clinical prostate cancer.41 Estradiol, in the presence of androgens, has been shown to stimulate carcinoma in situ and adenocarcinoma of the prostate in Noble rats.19,42–44 Estradiol is also capable of inducing precancerous lesions and prostate cancer in aging dogs.32 Thus, estrogenic stimulation with decreasing androgenic support contribute significantly to the pathogenesis of BPH, prostate dysplasia, and prostate cancer.36,45,46

**Estrogens and Selective Estrogen Receptor Modulators May Prevent Prostate Cancer**

**Phytoestrogens**

Evidence for a role of estrogen in prostatic carcinogenesis emerges from epidemiologic studies on diet. Phytoestrogens are nonsteroidal substances with weak estrogen agonistic activity. These weak estrogens are 1,000-fold less potent than estradiol but have similar relative affinity for ER as tamoxifen and selective estrogen receptor modulators (SERMs).47 Phytoestrogens essentially act like ER partial agonists/antagonists. There are 2 main classes of phytoestrogens: isoflavonoids and lignans. Phytoestrogens have been detected in human urine, plasma, semen, saliva, and prostate fluid samples.48 Soybean is a major source of isoflavonoids, mainly in the form of genistein. Soy is consumed daily in large amounts in China and Japan.48 A direct inverse correlation between serum levels of isoflavonoids and prostate cancer incidence has been observed.47 Phytoestrogens are thought to prevent prostate cancer by lowering 5α-reductase activity, increasing sex hormone-binding globulin, lowering free testosterone, decreasing tyrosine-specific protein kinase activity, and reducing p450 aromatase activity.47 Like weak estrogens, phytoestrogens have also been shown to lower cholesterol, decrease cardiovascular disease, prevent osteoporosis, and stop the progression of BPH.48

**Selective Estrogen Receptor Modulators**

Selective estrogen receptor modulators are generally considered “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 subtypes.49 Like phytoestrogens, SERMs possess the ability to suppress prostatic carcinogenesis. Unfortunately, several of the animal models of prostate cancer currently in use exhibit low positive control response rates and the role of
SERMs in prostatic carcinogenesis has remained inconclusive.\textsuperscript{50,51} One exception, however, is the TRAMP, a transgenic mouse model of prostate cancer.\textsuperscript{52} All animals that express the transgene eventually develop prostate cancer that mirrors human prostate cancer progression.\textsuperscript{53,54} The SERM GTx-006 (acapodene) was able to significantly reduce the incidence and increase the latency period of prostatic carcinogenesis in the transgenic adenocarcinoma of mouse prostate (TRAMP) (Raghow S and Steiner M, unpublished data). The mechanism of the chemopreventive effects of GTx-006 does not appear to be through suppression of transgenic expression of large Tag. GTx-006 is currently being studied in human clinical trials for the chemoprevention of prostate cancer. Thus, GTx-006 studies in the TRAMP model support the contention that SERMs, as a class, demonstrate chemopreventive activity against prostate cancer.

Accordingly, another SERM that may be useful in the chemoprevention of prostate cancer is arzoxifene ([6-hydroxy-3-[4-[(1-piperidinyl)ethoxy]phenoxyl]-2-(4-methoxy-phenyl)]benzothiophene) hydrochloride (LY353381·HCl). Arzoxifene is a SERM that, in preclinical models, exerts potent estrogen antagonist activity in mammary and uterine tissue while acting as an estrogen agonist to maintain bone density\textsuperscript{55} and lower serum cholesterol.\textsuperscript{56} Arzoxifene has demonstrated antitumor activity and clinical benefit and was well tolerated in phase 2 randomized double-blinded trials in women with locally advanced and metastatic breast cancer (unpublished data). Evidence for potential use of arzoxifene in prostate cancer chemoprevention comes from in vivo therapeutic studies with the compound against the androgen-sensitive LNCaP human prostate cancer xenograft model (unpublished data). Arzoxifene treatment of immunodeficient murine hosts produced marked inhibition of LNCaP tumor growth and time to disease progression. These antitumor effects on LNCaP xenografts were observed in the absence of estrogen-mediated reductions of host accessory sex organ and testicular weights. To the extent that the LNCaP model represents the phenotype of early hormone-sensitive disease, arzoxifene may be a useful chemotherapeutic agent, and with further studies perhaps may prove a chemopreventive agent, for prostate cancer.

**MECHANISMS OF CHEMOPREVENTION**

Estrogen stimulates cellular proliferation through ER by inducing local production of stimulatory peptide growth factors including transforming growth factor-\(\alpha\) (TGF-\(\alpha\)), insulin-like growth factor (IGF), and epidermal growth factor (EGF) and by inhibiting the local expression of growth inhibitory factors like transforming growth factor-\(\beta\) (TGF-\(\beta\)).\textsuperscript{57,58} Selective estrogen receptor modulators would therefore be expected to act at the cellular microenvironment level to decrease the amounts of these stimulatory growth factors and augment the production of TGF-\(\beta\). In addition, the antiproliferative effects of SERMs may be mediated by other intracellular signaling mechanisms, including binding and sequestration of calmodulin,\textsuperscript{59} inhibition of protein kinase C,\textsuperscript{60,61} and induction of p21\textsuperscript{62}waf1/cip1.\textsuperscript{63} Selective estrogen receptor modulators have the ability to bind to ER\(\alpha\) and ER\(\beta\) (1 and 2) to compete with estradiol and other estrogens for binding to ER in breast and prostate tissues.\textsuperscript{64,65,66,67} Formation of SERM-ER complexes results in the local inactivation of the estrogen-regulated genes, thereby decreasing cellular proliferation.

**SELECTIVE ESTROGEN RECEPTOR MODULATORS AS CHEMOPREVENTIVE AGENTS**

The ideal chemopreventive agent must have minimal or no side effects or toxicity to be accepted by otherwise healthy men who are at risk for development of prostate cancer. Selective estrogen receptor modulators, including arzoxifene, do not inhibit 5\(\alpha\)-reductase activity or testicular 17\(\alpha\)-hydroxy/C17,20-lyase activities. Chronic administration of high-dose arzoxifene in male rats, mice, and primates did not result in gynecomastia, or histopathologic changes in Leydig or Sertoli cell populations. Testicular and adrenal weights also were not significantly changed. With long-term treatment, SERMs had no significant physiologic effects on prolactin, luteinizing hormone, or follicle-stimulating hormone secretion.\textsuperscript{68} Thus, SERMs have been shown to have either no or only minimal effects on male reproduction. Because androgen levels are not affected, libido and sexual function should remain unchanged.

The first-generation SERM, tamoxifen, has been associated with a higher incidence of endometrial cancers.\textsuperscript{69} Tamoxifen increases the risk of endometrial cancer by forming DNA adducts suggesting that tamoxifen may function as an alkylating agent in the endometrium.\textsuperscript{70,71} A close correlation exists between the potential of a given compound to induce DNA-DNA adducts (DNA damage) and its neoplastic effects. Other SERMs, like GTx-006 or arzoxifene (unpublished results) do not form DNA adducts.\textsuperscript{70,71} Nonetheless, in general, SERMs have the same efficacy as the naturally occurring phytoestrogens that have been consumed by Asians for thousands of years.

In summary, SERMs (GTx-006 and arzoxifene) have many features that make them attractive candidates for prostate cancer chemoprevention.
They demonstrate chemopreventive activity against prostate cancer in different animal models of the disease and have a high safety profile when given chronically in animals. Moreover, SERMs have other beneficial effects. They prevent osteoporosis, lower serum lipid levels, reduce the rates of myocardial infarction, and may suppress the progression of BPH. The true benefit and efficacy of SERMs against prostate cancer, however, will ultimately be demonstrated only by conducting well-defined human clinical trials.

REFERENCES

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

Sharan Raghow,1 Emin Kuliyev, Marjorie Steakley, Norman Greenberg, and Mitchell S. Steiner

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 presented at 17 weeks of age, and by 33 weeks, all of the animals developed palpable tumors. In the high-dose flutamide group, however, tumors did not appear until 24 weeks, a lag of 7 weeks, and by 34 weeks, 42% of the animals were still tumor free. The period of time at which 50% of the animals had tumors was 33 weeks in the high-dose flutamide group, 24.5 weeks in the low-dose flutamide group, and 24.5 weeks in the placebo group. The difference between the placebo and high-dose flutamide groups was statistically significant (log rank, P = 0.0036; Wilcoxon's statistical analysis, P = 0.0060).

Tumors from high-dose flutamide-treated animals were more differentiated and retained much of the normal glandular architecture compared with those of the placebo group, whose tumors consisted of sheets of poorly differentiated cells. The expression of T antigen in the prostate tissues of flutamide-treated animals (at 10 weeks) was lower than that in the comparable placebo-treated group. Flutamide had the ability to suppress T antigen-driven carcinogenesis, resulting in a significant decrease in the incidence of prostate cancer and an increase in the latency period of prostate cancer in TRAMP mice.

INTRODUCTION

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

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

The study of prostate cancer chemoprevention has been hindered by the lack of appropriate animal models. Recently, a unique animal model known as the TRAMP(2) model of prostate cancer has been described (6, 7). In TRAMP mice, targeted expression of Tag driven by the prostate-specific promoter PB leads to transformation of cells in the prostate. This animal model has several advantages over the currently existing models: (a) the tumors occur with 100% frequency; (b) the mice develop prostatic epithelial hyperplasia and PIN, a premalignant lesion, as early as 10 weeks and develop invasive adenocarcinoma around 18 weeks of age; (c) the mice spontaneously develop 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 expeditious 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. The study of prostate cancer chemoprevention has been hindered by the lack of appropriate animal models. Recently, a unique animal model known as the TRAMP(2) model of prostate cancer has been described (6, 7). In TRAMP mice, targeted expression of Tag driven by the prostate-specific promoter PB leads to transformation of cells in the prostate. This animal model has several advantages over the currently existing models: (a) the tumors occur with 100% frequency; (b) the mice develop prostatic epithelial hyperplasia and PIN, a premalignant lesion, as early as 10 weeks and develop invasive adenocarcinoma around 18 weeks of age; (c) the mice spontaneously develop 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 expeditious evaluation of potential chemopreventive agents.

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

Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 weeks of age, snap-frozen in liquid nitrogen, processed in a Shandon-Lipshaw tissue processor, and embedded in paraffin. Tissue sections (4-μm thick) were stained with H&E for histological evaluation.

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

Western Blot Analyses. Ten cross-bred Tag-positive male pups (5 per group) were treated with either placebo or flutamide pellets at 4 weeks of age. Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 weeks of age, snap-frozen in liquid N2, and stored at -80°C. Tissue lysates were prepared using radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris (pH 7.5)) containing a mixture of protease inhibitors (Pefabloc, aprotinin, bestatin, leupeptin, and pepstatin) and the phosphatase inhibitor Na4VO4 (10 mM). The homogenate was centrifuged at 14,000 × g at 4°C for 10 min, and lysates were stored at -80°C until use.

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

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 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 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.

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&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0006&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 level of significance.

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

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

Fig. 3. Effect of flutamide on prostate tumor development in the TRAMP model. H&E stain; magnification, ×66.

Wild-type mouse ventral prostate-17wk
TRAMP ventral prostate-17wk
Placebo-treated prostate-15wk
Flutamide-treated prostate-15wk

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 5α-reductase inhibitor, is currently being investigated as an agent to prevent prostate cancer in the National Cancer Institute-sponsored Prostate Cancer Prevention Trial. However, its ability to prevent prostate cancer in animals has
never been demonstrated. Consequently, other agents with 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
at 4 weeks age (this study) versus castration at 12 weeks age (5).
These data imply that androgen ablation with flutamide during the
early stage of carcinogenesis may be an effective chemopreventive
measure against prostate cancer. It is conceivable that castration sets
up an environment conducive to more aggressive androgen-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-ß1 has been shown to reduce
mammary tumor formation in transgenic mice. This suggests that the chemopreventive effects
of flutamide might be mediated through TGF-ß1.

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
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 protein for its assigned role as the gatekeeper of cellular growth and division (21), which results in the delay of prostate cancer. The SV40 large Tag binds and inactivates p53 protein (22), and the loss of tumor suppressor wild-type p53 and Rb genes has been implicated in the development of prostate cancer (23, 24). In the TRAMP model, Tag expression leads to abrogation of p53 and Rb functions, 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 human subjects, far less than the currently prescribed 750 mg/day.

REFERENCES

Toremifene Prevents Prostate Cancer in the Transgenic Adenocarcinoma of Mouse Prostate Model

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ABSTRACT

The chemopreventive efficacy of toremifene, an antiestrogen, was evaluated in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. TRAMP mice were segregated into three groups: (a) the low-dose toremifene group (6.6 mg/kg/day); (b) the high-dose toremifene group (33 mg/kg/day); and (c) the control placebo group. Efficacy of treatment was measured by the absence of palpable tumor. To extend these studies using more sensitive techniques, TRAMP mice were then treated with placebo, flutamide (an antiandrogen; 33 mg/kg/day), or toremifene (10 mg/kg/day). Animals from each treatment group were sacrificed at 7, 10, 15, 20, 25, and 30 weeks of age, and prostate tissues and seminal vesicles were harvested. Tissues from animals (n = 5) in each group were evaluated by wholemount dissections of genitourinary tracts, histology, immunohistochemistry, and Western blot analyses. Blood was pooled per group to measure estradiol and testosterone hormonal levels. Tumors formed at week 17 in the placebo group (n = 10), at week 21 in the high-dose toremifene group (n = 12), and at week 29 in the low-dose toremifene group (n = 12). This represents an increased tumor latency of up to 12 weeks. By 33 weeks, all animals in the placebo group had tumors compared with only 35% of the animals treated with toremifene. Although both flutamide and toremifene decreased tumor incidence compared with the placebo, toremifene was more effective than flutamide. High-grade prostatic intraepithelial neoplasia was observed in animals in the placebo group, but not in animals treated with toremifene. Moreover, toremifene-treated animals had prolonged survival compared with placebo-treated animals. By 33 weeks of age, 100% of the placebo-treated animals had developed palpable tumors and died, whereas 60% of the toremifene-treated animals were tumor-free. T antigen levels in the prostate of toremifene-treated animals were similar to those of placebo-treated, age-matched animals. Whereas serum estradiol levels remained unchanged, the total free testosterone levels were elevated in the toremifene-treated group. Toremifene treatment did not affect androgen receptor expression. Because toremifene prevented prostate cancer in a milieu of elevated free testosterone levels with no change in prostate androgen receptor expression, the mechanism of toremifene’s chemopreventive activity may be through nonandrogenic pathways, such as estrogen receptor signaling.

INTRODUCTION

Prostate cancer is the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer deaths in men (1). Changes in androgen and estrogen levels with age are thought to be involved in prostate cancer because its incidence rises sharply with age (2). The focus of chemoprevention is not on the treatment of the disease (cancer) but rather on the oncogenic process [carcinogenesis (3)]. High-grade PIN4 is considered a precursor of adenocarcinoma of the prostate because about 60% of men with high-grade PIN develop prostate cancer within 2 years (4, 5). Androgen deprivation by flutamide and LHRH agonists, but not by finasteride, reduced high-grade PIN (5–7). Unfortunately, the serious side effects of testosterone-lowering drugs are not acceptable to men without prostate cancer.

Increasing serum estrogens and decreasing serum androgens and 5α-reductase activity with age lead to stromal hyperproliferation in the prostate (2). Rising estrogens appear to increase sensitivity of the prostate tissue to androgens by up-regulation of the AR (8–10). Estradiol in the presence of androgens has been shown to stimulate carcinoma in situ and adenocarcinoma of the prostate in Noble rats (11–14). Estradiol is also capable of inducing high-grade PIN and prostate cancer in the aging dog (2, 15). Thus, estrogen stimulation with decreasing androgen levels contributes to the genesis of prostatic dysplasia and subsequent prostate cancer (16–18).

Both the prostatic stroma and epithelium express ERs, and estrogens are important for prostate growth (19, 20). Recently, a new ER, ERβ, was cloned from a rat prostatic cDNA library and is present in murine and human prostates (21–24). Consequently, the previous ER is now designated as ERα. ERα and ERβ are highly homologous, have similar affinity for estradiol, and can hetero- or homodimerize to form a signaling dimeric complex (21, 22). Although estradiol activates both ERα and ERβ, ERα stimulates transcription and cellular proliferation, whereas ERβ quenches ERα activation (25). ERα is localized predominantly in the prostatic stroma (26), whereas ERβ is found in the secretory epithelial cells of the prostate (21, 22).

In the TRAMP model, the PB-Tag transgene is expressed specifically in the epithelial cells of the prostate. The probasin promoter contains an ARE. All TRAMP mice express the transgene in an androgen-dependent manner and eventually develop prostate cancer that mirrors human prostate cancer progression (27, 28). The TRAMP model has several advantages over currently existing models: (a) mice develop progressive forms of PIN as early as 10 weeks and develop invasive adenocarcinoma by 18 weeks of age; (b) metastatic spread of prostate cancer in TRAMP mice to lymph node, lung, kidney, adrenal gland, and bone resembles human disease; (c) development and progression of prostate cancer can be followed within a relatively short period of 10–30 weeks; (d) prostate tumors arise with 100% frequency; and (e) animals may be screened for the presence of the prostate cancer transgene before the onset of clinical prostate cancer. Thus, TRAMP transgenic mice represent a reliable model to directly test the efficacy of chemopreventive agents that may alter prostate carcinogenesis.

SERMs are structurally diverse nonsteroidal compounds that functionally mimic estradiol in their action but also possess cancer-suppressing activity. Tamoxifen, a SERM, has been widely used to treat breast cancer. Toremifene is a chlorinated derivative of tamoxifen that lacks the DNA adduct forming ability of tamoxifen and has lower genotoxicity than tamoxifen (29–31). Toremifene inhibited 7,12-dimethylbenz(a)anthracene-induced rat mammary cancer (32). Toremifene has been used for breast cancer treatment in 27 countries and used for as long as 13 years in Finland (33). Consequently,
Toremifene was selected as the SERM of choice to study its chemopreventive efficacy in the TRAMP model. We report that toremifene suppressed the development of high-grade PIN, decreased prostate cancer incidence, and increased survival.

MATERIALS AND METHODS

The animal experimental protocol was approved by an institutional animal experimentation review board and followed the NIH guidelines for proper and humane use of animals. The TRAMP (C57BL/6 PB-Tag) transgenic mice were cross-bred 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 this study. Toremifene citrate 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 in PB-Tag mice (4 weeks of age; average weight, 14 g) anesthetized with metofane (Mallinckrodt, Mundelein, IL) as described previously (34).

For palpable tumor study, the number of animals and the doses of toremifene used were based on published animal data (32). Three groups of 10–12 animals each received a 90-day-release drug pellet of either low-dose toremifene (6.6 mg/kg/day) or high-dose toremifene (33 mg/kg/day) or a placebo. Each treated animal received supplemental dosages at 90-day intervals. Starting at the age of 10 weeks, animals were evaluated weekly for the absence or presence of a palpable tumor. The differences between treatment groups were compared by Fisher's exact test and Wilcoxon's rank-sum test for statistical analysis. All Ps were two-sided.

Because both 6.6 and 33 mg/kg/day of toremifene showed chemopreventive efficacy, we selected an intermediate dose (10 mg/kg/day toremifene) for longitudinal cohort analysis to determine the effects of toremifene on high-grade PIN and tumor incidence. Three cohorts of animals (70 animals/ cohort, 10 animals/time point) were treated with placebo, flutamide (33 mg/kg/day), or toremifene (10 mg/kg/day) pellets starting at 4 weeks of age. Animals from each group were sacrificed at 7, 10, 15, 20, 25, 30, and 33 weeks of age. Tissues from mice (n ≥ 5) were evaluated by whole mount dissections of genitourinary tracts, histology, immunohistochemistry, and Western blot. Blood was pooled, and serum was stored at -20°C for assay of hormone levels. Serum testosterone and estradiol levels were assayed using the EIA (Diagnostic Systems Laboratories, Inc. Houston, TX). Values for the sample analyte were derived by interpolation using standards available with the kit.

Whole mount analysis and histology. Wholemounts of ventral prostates (7, 10, 15, 20, and 30 weeks of age) and seminal vesicles (7, 10, 15, and 20 weeks of age) were examined under a dark-field dissection microscope (Olympus SZH stereo-fitted with an Olympus camera). For histological evaluation, prostate tissues were harvested, fixed overnight in 10% buffered formalin, processed in a Shandon-Lipshaw tissue processor, and embedded in paraffin. Tissues from mice (n ≥ 5) were evaluated by whole mount dissections of genitourinary tracts, histology, immunohistochemistry, and Western blot.

Immunohistochemistry. Paraffinized prostate tissue sections (4 μm) were obtained from 7-, 10-, and 15-week-old animals treated with placebo, flutamide, or toremifene. Antigen retrieval was performed using the Trilogy (Cell Marque, Austin, TX) method according to the manufacturer's protocol. Tissues were incubated with the anti-Tag primary antibody (Pabl10 mouse monoclonal antibody; 1:150; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. For Tag immunostaining, the M.O.M. kit (Vector Laboratories) was used to eliminate all nonspecific staining attributable to mouse monoclonal primary antibody. Tissue sections were treated with the secondary antibody (goat antimouse IgG; SC2039; Santa Cruz Biotechnology) for 10 min. To quench endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in water and then rinsed in PBS for 5 min. ABC reagent (Vectastain, Vector Laboratories) was applied for 5 min, and the sections were treated with Nova Red substrate (Vector Laboratories) for 10 min and rinsed with running tap water for 5 min. Harris Hematoxylin was used as counterstain. Samples were then dehydrated through a series of alcohol dilutions, cleared through xylene, and mounted on slides using Cytoseal-60 (Stephens Scientific, Kalamazoo, MI).

Western blot analyses. Cross-bred Tag-positive male pups (5 pups/group) were treated with either placebo or toremifene (10 mg/kg/day) pellets at 4 weeks of age. Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 and 15 weeks of age, snap-frozen in liquid N2, and stored at -80°C. Western blot analysis of tissue lysates was performed as described previously (34). TRAMP prostate tumor tissue was used as positive control. Blots were blocked overnight at 4°C in BLOTTO and sequentially reacted with the large Tag primary antibody and HRP-conjugated secondary antibody. The AR expression in placebo and toremifene-treated TRAMP mice (15 and 20 weeks of age) was analyzed by Western blot using primary antibody (rabbit polyclonal antibody SC 816; Santa Cruz Biotechnology) and HRP-conjugated secondary antibody. To normalize the results, Tag and AR expression blots were stripped and reacted with antiactin mouse monoclonal primary antibody (Chemicon, Temecula, CA) followed by HRP-conjugated goat antiamine secondary antibody.

RESULTS

Toremifene Suppresses the Occurrence of Palpable Tumors in TRAMP Mice. Prostate tumors were first palpable in the placebo group (n = 10) by week 17, in the low-dose (6.6 mg/kg/day) toremifene group (n = 12) by week 29, and in the high-dose (33 mg/kg/day) toremifene group (n = 12) by week 21 (Fig. 1). Hence, toremifene increased the latency time of palpable prostate cancer by up to 12 weeks. Tumors were palpable in 25% of the animals by week 18 in the placebo group and by 33–34 weeks in the high- and low-dose toremifene-treated groups. By 34 weeks, 100% of the placebo-treated animals had palpable tumors compared with 35% of the toremifene-treated animals. Differences in the presence of palpable tumors between low- and high-dose toremifene-treated groups versus placebo groups were significant by both log-rank and Wilcoxon's statistical analysis (P < 0.0003, low-dose toremifene; P < 0.00017, high-dose toremifene). The incidence of palpable tumors was not significantly different in the high- and low-dose toremifene-treated groups. Toremifene-treated animals also had greater survival rates than the placebo-treated group. By 33 weeks, 100% of the placebo-treated animals had developed palpable tumors and died, whereas 60% of the high- and low-dose toremifene-treated animals had no palpable tumors and were still alive. Furthermore, toremifene-treated mice did not exhibit loss of appetite or weight. No skin lesions or preening behavior resulted from the drug. Thus, treatment with either the high or low doses of toremifene significantly decreased the incidence and
increased the latency period of palpable prostate tumors and prolonged survival.

**Toremifene Prevents the Formation and Progression of Prostate Cancer.** The ability of toremifene to suppress prostate carcinogenesis was further investigated by wholemount analysis and histological evaluation as more sensitive measures of tumorigenesis. Genitourinary tracts from placebo- and toremifene-treated TRAMP mice (n ≥ 5) sacrificed at 7, 10, 15, 20, and 30 weeks were examined. Wholemount analysis of murine ventral prostates (Fig. 2) revealed that invasive prostate tumors resulting in fused ducts were detectable as early as 15 weeks of age (Fig. 2C) and that tumors were present in 100% of prostates from the placebo group by 30 weeks of age. In contrast, the toremifene-treated group had no evidence of fused ducts; the ducts remained distinct and delicate up to 30 weeks of age in 72% of the animals (Fig. 2, I and J). Interestingly, toremifene also reduced seminal vesicle size compared with the placebo group (Fig. 3).

Histological sections were obtained from the ventral prostate of 17 week-old wild-type mice as a normal control. The wild-type ventral prostate had delicate epithelial ducts with sparse intervening stroma (Fig. 4A). In contrast, ventral prostate sections from placebo control TRAMP mice (Fig. 4B) had complete replacement of the normal prostate ductal structures by poorly differentiated anaplastic cells by 17 weeks. Ventral prostate section contained high-grade PIN in 7- and 15-week-old placebo-treated animals (Fig. 4, C and D), but not in age-matched toremifene-treated animals (Fig. 4, E and F). Toremifene treatment of TRAMP mice maintained normal prostatic epithelial ductal architecture (Fig. 4A).

Table 1 shows the effects of placebo, flutamide, and toremifene on prostate oncogenesis in the TRAMP model. Placebo-treated mice uniformly developed prostate tumors by 15–20 weeks of age, whereas toremifene-treated animals had a reduction in the development of prostate cancer for up to 33 weeks. By 15 and 20 weeks of age, 50% and 100% of placebo-treated animals had detectable prostate cancer, respectively. In contrast, by 15 and 20 weeks of age, 0% and only 14% of toremifene-treated animals had evidence of prostate cancer, respectively. The time it took for 50% of placebo-treated animals to develop tumors was 15 weeks; only 43% of the toremifene-treated animals had tumors at 33 weeks. Notably, compared with placebo-treated animals, the tumor incidence in TRAMP mice was about 50% lower with flutamide treatment (43%, 50%, and 57%) and about 75% lower with toremifene treatment (14%, 20%, and 28%) at the same ages. By χ² analysis with an overall level of significance of 0.05 and a power of study of 0.80, the data at 30 weeks showed statistical significance (P < 0.031). We did not perform repeated measure ANOVA because the size of the tumor was not measured. These data further confirm that even with a more sensitive assessment of tumorigenicity, toremifene had significant chemopreventive activity. In fact, toremifene is a more potent chemopreventive agent for prostate cancer than flutamide.

**Toremifene Affects Serum Hormonal Levels.** The free and total serum testosterone and serum estradiol levels were measured using the EIAs. Although toremifene did not affect serum estradiol levels, total serum testosterone levels in treated mice were elevated at 10–15 weeks and returned to levels that were comparable with those of placebo-treated animals by 20–30 weeks. In contrast, the level of free serum testosterone remained elevated from 10–30 weeks of age compared with that in 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 for up to 30 weeks.

**The Large Tag Transgene in the TRAMP Is Not Down-Regulated by Toremifene.** 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 the probasin promoter activity should be inhibited. Consequently, large Tag expression was determined by Western blot analysis, and representative data are shown (Fig. 5). The large Tag oncoprotein was present in TRAMP mice...
Toremifene as a Chemopreventive Agent

Fig. 4. Histological evaluation of TRAMP prostate tissue. A and B, histology of wild-type mouse ventral prostate and TRAMP ventral prostate/tumor at 17 weeks of age. H&E stain; magnification, ×66. C and D, H&E stain of 7- and 15-week-old placebo-treated prostate with PIN and early-stage tumor. E and F, 7- and 15-week-old toremifene-treated prostate [magnification, ×33 (C and E) and ×13.2 (D and F)].

prostate tumor tissue and in tissues obtained from TRAMP mice at 10 and 15 weeks of age treated with and without toremifene (Fig. 5, A and B). Moreover, the level of large Tag was relatively higher in the toremifene-treated prostate than it was in the placebo-treated prostate.

These observations on large Tag expression were confirmed by immunohistochemical staining of 7-, 10-, and 15-week-old prostate tissues from placebo-treated (Fig. 6, A–C) and toremifene-treated animals (Fig. 6 G–I). Prostate tissues from age-matched flutamide-treated animals (Fig. 6, D–F) were used to compare the relative effects of an antiandrogen, flutamide, with the antiestrogen toremifene on large Tag expression. Animals treated with flutamide had no detectable large Tag protein, whereas toremifene and placebo-treated prostate sections had similar amounts of large Tag protein. These studies suggest that flutamide down-regulated the expression of large Tag, which accounted for its prostate chemopreventive activity. In contrast, toremifene did not alter large Tag expression, suggesting that the mechanism of toremifene’s chemopreventive activity against prostate cancer is not related to large Tag expression but rather to direct suppression of carcinogenesis.

AR Levels in the TRAMP Prostate Are Not Down-Regulated by Toremifene. AR was present in the TRAMP tumor tissue. Prostatic AR levels were higher in hybrid TRAMP (TRAMP × FVB) transgenic mice compared with nontransgenic mouse with the same genetic background (C57/BL6 × FVB; Fig. 7). By Western blot analysis,
Toremifene treatment did not alter the expression evidenced by the reduced seminal vesicle size in the face of elevated serum testosterone levels. Thus, the mechanism of toremifene chemopreventive action because (a) toremifene did not suppress the ARE-dependent induction of animals with tumor is shown. The number of animals with tumor/actual number of animals sacrificed is shown in parentheses.

**DISCUSSION**

The TRAMP model is ideally suited to study chemoprevention because 100% of mice develop prostate cancer that mirrors the human form of the disease (27, 28). Toremifene treatment significantly reduced the incidence of prostate cancer in TRAMP mice. This is the first report on the chemopreventive 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 to flutamide, toremifene inhibits prostate carcinogenesis independent of its effect on large Tag.

Toremifene was well tolerated at the doses used in our study, with no obvious signs of toxicity. According to an earlier report (32), one significant increase in the latency period of prostate cancer observed in toremifene-treated animals suggests that toremifene is able to suppress carcinogenesis. In contrast to flutamide, toremifene inhibits prostate carcinogenesis independent of its effect on large Tag.

Our study, toremifene treatment of mice resulted in elevated testosterone levels. Elevated testosterone levels with tamoxifen treatment have been reported (35). Toremifene, like tamoxifen, increases circulating testosterone levels by interfering with the hypothalamus-pituitary-gonadal axis (36). Toremifene blunts the pituitary's ability to suppress LH/H secretion in response to testosterone. However, toremifene does block the local tissue effects of testosterone action, as evidenced by the reduced seminal vesicle size in the face of elevated serum testosterone in our study.

Fig. 5. Western blot analysis of large Tag levels. A. representative Western blots using prostate tissue lysates (40 μg protein/lane) of 15-week-old placebo- or toremifene-treated mice; top panels, membrane probed with anti-large Tag mouse monoclonal IgG; bottom panels, membrane reprobed with antiantigen mouse monoclonal IgG as internal control. Prostate tumor from a 10-week-old untreated TRAMP mouse was used as positive control. B. Western blot of prostate tissue lysates from 10-week-old mice. Top panel, membrane probed with anti-large Tag mouse monoclonal IgG; bottom panel, membrane reprobed with antiantigen mouse monoclonal IgG as internal control.
cells (14, 21–24). Older ERβ knockout (β-ERKO) mice develop prostate hyperplasia, supporting the contention that ERβ normally suppresses prostate epithelial proliferation (38). In contrast, ERα, not ERβ, is the predominant ER expressed in the female reproductive system (21, 22). We believe that earlier data on the role of ER in the prostate must be reevaluated because those studies were unable to distinguish between ERα and ERβ. SERMs can bind to ERα and ERβ and compete with estradiol and other estrogens in breast and prostate tissue (22, 25, 39–41). Signals emanating from SERM-ER interaction result in the inactivation of estrogen-regulated genes, leading to inhibition of cellular proliferation. Recently, Wang et al. (42) have reported that ERα is critical for prostate carcinogenesis because wild-type mice treated with testosterone and estradiol sequentially developed prostate hyperplasia, high-grade PIN, and prostate adenocarcinoma. In contrast, ERα knockout transgenic mice with identical hormone regimen had epithelial hyperplasia but did not exhibit high-grade PIN or prostate cancer. This is reminiscent of our results with TRAMP. The untreated TRAMP mice develop hyperplasia, high-grade PIN, and prostate cancer, and treatment with toremifene reduced high-grade PIN and prostate cancer. We speculate that toremifene may mediate its actions via ERα because a dynamic modulation of ERs is seen in the prostates of toremifene-treated mice. The antiproliferative effects of SERMs may also be mediated by other intracellular signaling mechanisms including binding and sequestration of calmodulin (43), inhibition of protein kinase C (44, 45), and induction of p21^{waf1/cip1} (45). Nonetheless, the exact mechanism of toremifene-mediated chemoprevention of prostate cancer remains to be elucidated.

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REFERENCES


MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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