

Award Number: DAMD17-98-1-8642

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

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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20001204 039

REPORT DOCUMENTATION PAGE*Form Approved*
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1999	3. REPORT TYPE AND DATES COVERED Annual (30 Sep 98 - 29 Sep 99)	
4. TITLE AND SUBTITLE Role of the TGF-B 1 in the Prevention of Prostate Cancer			5. FUNDING NUMBERS DAMD17-98-1-8642	
6. AUTHOR(S) Mitchell Steiner, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Tennessee Memphis, Tennessee 38163 e-mail: msteiner@utmem1.utmen.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Our preliminary data showed that antiestrogen (toremifene) and antiandrogen (flutamide) prevented cancer in the TRAMP transgenic mouse model. Moreover, retinoids have been reported to prevent prostate cancer. However, the mechanism of prostate cancer prevention by these agents is unclear. We hypothesized that these agents inhibit prostate carcinogenesis through stimulation of TGFβ production. This hypothesis is being tested tested in TRAMP transgenic mice through two specific aims: 1) whether the chemopreventive biologic effects of antiandrogens, antiestrogens, and retinoic acid are mediated by TGFβ1 in the TRAMP model, 2) whether prostate cancer may be prevented in the TRAMP model at the genetic level by crossbreeding with transgenic mice engineered to overexpress TGFβ1 in the prostate. By detailed wholmount and histologic analyses flutamide, and toremifene were able to delay onset of prostate cancer. 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 affect on large T antigen expression. The retinoic acid derivative did not inhibit the onset of prostate cancer and as such did not demonstrate chemopreventive activity. Transgenic mice engineered to overexpress prostatic TGFβ1 had smaller prostates.				
14. SUBJECT TERMS Prostate Cancer			15. NUMBER OF PAGES 49	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION

One third of all men over 50 years of age have a latent form of prostate cancer that may develop into potentially life-threatening clinical prostate cancer. Prostate cancer is a multi-step process that represents the accumulation of genetic mutations resulting in unregulated cellular proliferation.¹ Evidence suggests that modifications in environmental, dietary, endocrine, or genetic factors may help prevent prostate cancer. As the peptide growth factor TGF β has the ability to inhibit normal epithelial cell growth, then agents that can stimulate TGF β ²⁻⁷ production would be expected to slow the transformation of latent prostate cancer to clinical prostate cancer. Our preliminary data showed that antiestrogen (toremifene) and antiandrogen (flutamide) prevented cancer in the TRAMP transgenic mouse⁸ model. Moreover, retinoids have also been reported to prevent prostate cancer.⁹⁻¹² However, the mechanism of prostate cancer prevention by these agents is unclear. We hypothesize that these agents stimulate TGF β production that in turn inhibits prostate carcinogenesis by preventing the activation of latent prostate cancer. This hypothesis will be 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 TGF β 1 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 TGF β 1 in the prostate. Hence, identification of a chemopreventive agent with proven biologic efficacy in a exciting new prostate animal model with appropriate surrogate markers of carcinogenesis should have important implications for human prostate cancer chemoprevention clinical trials. Because of the high incidence and mortality of prostate cancer, it is imperative to develop chemoprevention strategies against this devastating disease.

BODY

Task 1. To determine whether the chemopreventive biologic effects of antiandrogens, antiestrogens, and retinoic acid is mediated by TGF β 1 in the TRAMP model

A. 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. Wholemound prostate dissections
3. To evaluate changes in serum androgens and estrogens with chemoprevention (**months 1-12**)

Task 1 Status

A. The hybrid TRAMP mice (C57/BL6 PBTtag x FVB-wt) litters were screened for the SV40 large T-antigen transgene and at 4 weeks age, the positive males were implanted with the drug pellets. During the past 12 months, the emphasis was on the wholemount studies and collecting the appropriate tissues for histology as well as molecular studies. For each time point, 5-7 animals each were treated with either placebo, or flutamide (30mg/kg/day), or toremifene (10 mg/kg/day) or the 9cRA(the 9cis Retinoic acid derivative MDI 301). Animals were sacrificed at 7, 10, 15, 20, and 25 weeks of the proposed schedule and tissues (ventral prostate, dorsolateral prostate, anterior prostate and seminal vesicles) harvested for morphology (wholemounts), for histology (formalin-fixed and paraffin-embedded), and molecular studies (frozen in liquid nitrogen and stored at -80° C. Blood was also collected and the serum stored frozen for hormone analyses.

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 (Table 1). Work is in progress to include the 40- and 50-weeks time-points in the due course.

Wholemount studies using dark-field microscopy was done to ascertain the absence/presence of a nonpalpable tumor, to locate the origin of the tumor and any change in the ductal development. To date the ventral prostate, anterior prostate, and seminal vesicle wholemounts for 7, 10, 15, 20, and 25 weeks for the various groups have been completed. Dissection of the animals for wholemount studies confirmed the time of palpable tumor appearance assessed in our pilot study i.e. palpable tumors in nontreated TRAMP mice appear at 15-20 weeks of age. The chemopreventive delay of prostatic tumors by flutamide and toremifene beyond 20 weeks was also quite apparent (Fig. 1). The seminal vesicle size in the drug-treated animals was much smaller compared to the

palcebo, the effect of toremifene being much more pronounced than that of Flutamide (Fig. 2). However, the cis-retinoic acid derivative MDI 301 used in this study showed no chemopreventive effect. The MDI 301-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) and no change in the seminal vesicle size compared to the placebo (Fig. 3) and, (Fig. 4). Consequently, we decided to stop further retinoic acid treatment and sampling and our current focus is on the flutamide and toremifene groups.

Next, we will be studying the histological changes associated with initiation and progression of prostatic neoplasia and the mechanism of its delay by chemopreventive drugs. The first focus will be on the histology of 7, 10, 15, and 20 week samples. Since frank tumors appear by 15 weeks in the placebo-treated animals these samples should reveal any precancerous precursor markers of prostate adenocarcinoma such as PIN and help narrow down the window of efficacy of drug treatment. These studies will be done in parallel with studies of the relevant samples at the molecular level.

B. Studies were carried out to alleviate a primary but very important concern of the Reviewer A. (verbatim)"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".

In order to answer this question, animals were bred, screened and implanted with placebo, flutamide (antiandrogen) and toremifene (antiestrogen) pellets. The Western Blot and the chemiluminescence techniques were optimized and applied to measure the large-T Ag 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. These results indicate that the delay/inhibition of cancer initiation by flutamide is most likely mediated through inhibition of the SV40 T-antigen 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. With this data incorporated, the manuscript **"Efficacious chemoprevention of primary prostate cancer by flutamide in an autochthonous transgenic model"** has been submitted to Cancer Research in September, 1999 (attached).

Interestingly, however, the T-antigen levels in the toremifene-treated animals were significantly higher than in the placebo-treated animals. Thus, it seems that the mechanism of inhibitory effect of antiestrogens on prostate cancer development in the TRAMP model is different from that of the antiandrogens and is not through simply downregulation of the T-antigen. The next challenge is to dissect out this difference in mechanism of action at the histological and molecular level. **The manuscript detailing the results of toremifene efficacy is currently in preparation.**

C. Immunohistochemical methods for TGF α and TGF β 1-3 to be used in this project were developed and used to study expression of these growth factors in the human fetal prostate tissues. Reprint attached, **Raghow et al. "Immunohistochemical localization of transforming growth factor- α and transforming growth factor β during early human fetal prostate development". Journal of Urology, 1999; 162:509-513.** Tissues have been collected to apply these techniques. It is critical to wait until all tissues from all treatment group timepoints have been collected so that all the tissue sections may undergo immunohistochemistry together. This will minimize the inter- and intra- assay variability.

Task 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 prostate TGF β 1.

Task 2 Status

Characterization of the PBTGF β transgenic mice was done to evaluate the target-specificity of the transgene expression. Of the 7 transgenic mice (5 males and 2 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 2 presents a summary of the tissue-specific TGF β expression. It was observed that line T 2353 showed ventral prostate specific expression of TGF β , 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 T-2353 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 TGF β transgene was associated with reduction in the number of ductal glands and the size of the prostate. Next, these mice will be 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 TGF β 1 in the prostate. The progeny will be screened and those expressing both the large T-Ag and the TGF β transgenes will be studied using the guidelines in Task 2.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed by detailed wholemount and histologic analyses that antiandrogen, flutamide, and antiestrogen, toremifene were able to delay onset of prostate cancer
- 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 affect on large T antigen expression
- Retinoic acid (cis -Retinoic acid derivative MDI-301) did not inhibit the onset of prostate cancer and as such did not demonstrate chemopreventive activity
- Transgenic mice engineered to overexpress prostatic TGF β 1 had smaller prostates

REPORTABLE OUTCOMES

Abstracts

Raghow S, Kuliyeve E, Greenberg N and Steiner M. **Flutamide for chemoprevention of Cancer** presented at AUA 1999, Dallas, TX.

Raghow S, Steakley M and Steiner M. **Flutamide downregulates probasin promoter-driven expression of T-antigen in the TRAMP model of prostate cancer.** Submitted to AACR 2000, San Francisco, CA.

Manuscripts

Raghow S, Steakley M, Greenberg N and Steiner M. **Efficacious chemoprevention of primary prostate cancer by flutamide in an autochthonous transgenic model.** submitted to Cancer Research in September, 1999 (copy attached).

Raghow S, Steakley M and Steiner M. **Antiestrogen toremifene prevents primary prostate cancer in the transgenic model.** (in progress).

Raghow S and Steiner M. **Prostate-targeted overexpression of TGF β in a transgenic mouse model .** (in progress).

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. One potential mechanism may be through stimulation of TGF β . The implications of this work is that prostate carcinogenesis may be inhibited resulting in a decreased incidence of prostate cancer. Flutamide and toremifene should be considered for human prostate chemopreventive clinical trials if their toxicity is limited.

REFERENCES

1. Steiner MS, Satterwhite DJ, Moses HL. Molecular insights into altered cell cycle regulation and genitourinary malignancy. *Urol Oncol* 1995; 1:3-17.
2. Knabbe C, Klein H, Zugmaier G, Voigt KD. Hormonal regulation of transforming growth factor - β 2 expression in human prostate cancer. *J Steroid Biochem Molec Biol* 1993; 4:137-142.
3. Thompson TC. Growth factors and oncogenes in prostate cancer. *Cancer Cells* 1990; 2(11):345-354.
4. Rijnders AWM, van der Korput JAGM, Van Steenbrugge GJ, Romijn JC, Trapman J. Expression of cellular oncogenes in human prostatic carcinoma cell lines. *Biochem Biophys Res Comm* 1985; 132(2):548-554.
5. Pierce DF, Johnson MD, Matsui Y, et al. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF β 1. *Genes Dev* 1993; 7:2308-2317.
6. Pierce Jr, PF, Gorska AE, Chytil A, et al. Mammary tumor suppression by transforming growth factor β 1 transgene expression. *Proc Natl Acad Sci USA* 1995; 92:4254-4258.
7. Colletta AA, Wakefield LM, Howell FV, et al. Antiestrogens induce the secretion of active TGF β from human fetal fibroblasts. *Brit J Cancer* 1990; 62:405-409.
8. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci USA* 1995; 92:3439-3443.
9. Glick AB, McCune BK, Abdulkarem N, et al. Complex regulation of TGF β expression by retinoic acid in the vitamin A-deficient rat. *Dev* 1991; 111:1081-1086.
10. Sporn MB, Roberts AB. Interactions of retinoids and transforming growth factor β in regulation of cell differentiation and proliferation. *Mol Endocrinol* 1991; 5:3-7.
11. Falk LA, De Benedetti F, Lohrey N, et al. Induction of TGF β 1 protein production in retinoic acid treated HL-60 cells: Possible TGF β 1 mediated autocrine inhibition. *Blood* 1991; 77:1248-1255.
12. Gucev ZS, Youngman O, Kelley KM, Rosenfeld RG. Insulin-like growth factor binding protein 3 mediates retinoic acid and TGF β 2 induced growth factor inhibition in human breast cancer cells. *Cancer* 1996; 56:1545-1550.

Table 1. Prostate tumor development in the TRAMP mouse treated at age 4 weeks with either cRA, Flutamide, or Toremifene.

Treatment	20 wk	25 wk	30 wk	33 wk
Placebo	100%	83%	100%	all died
cRA	80%	80%	x	x
Toremifene	16%	20%	28%	*
Flutamide	43%	50%	57%	*

% of animals with a tumor; x, discontinued; * being continued.

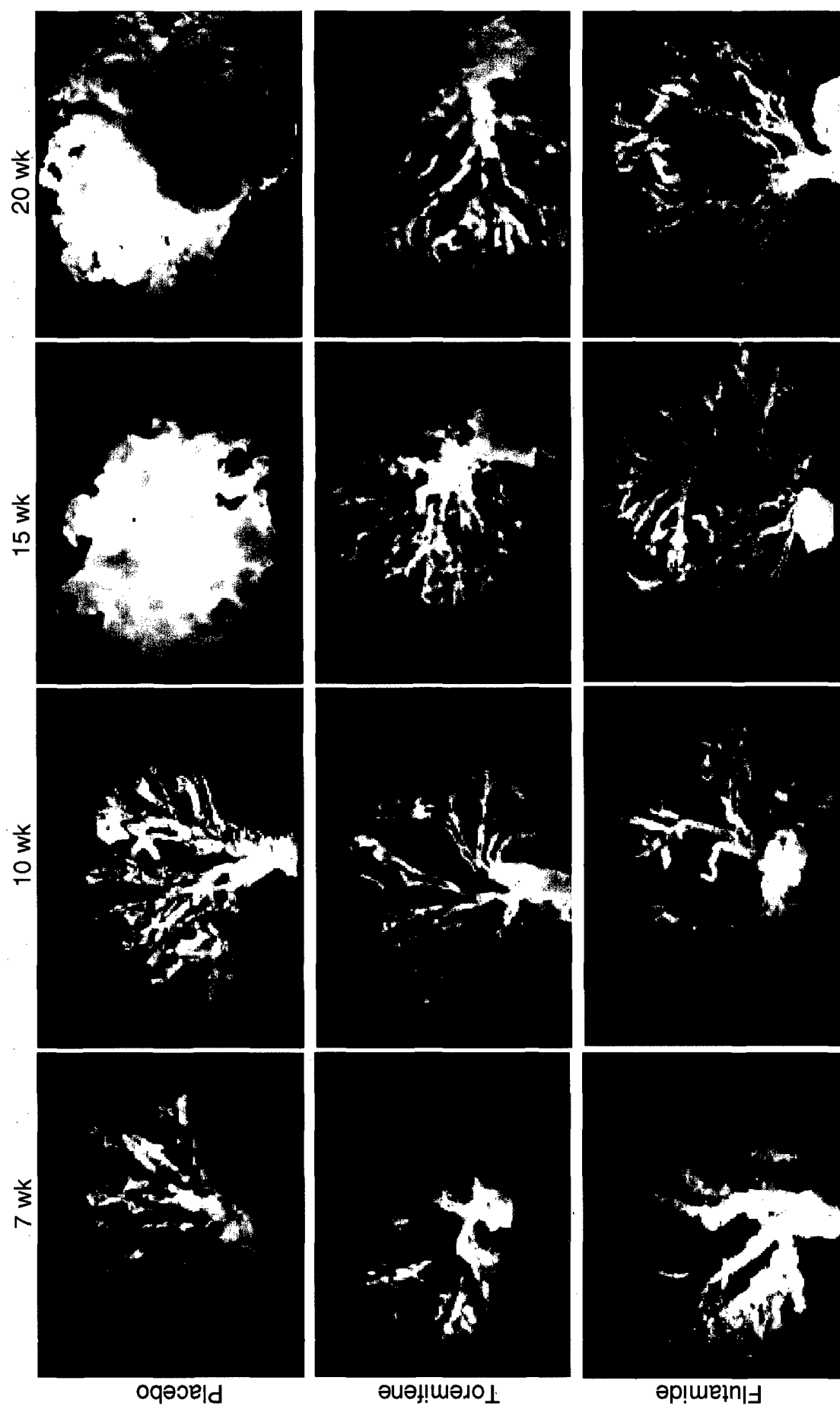


Fig. 1. Effect of Flutamide and Toremifene treatment vs. the Placebo on Ventral Prostate development in the TRAMP mouse.

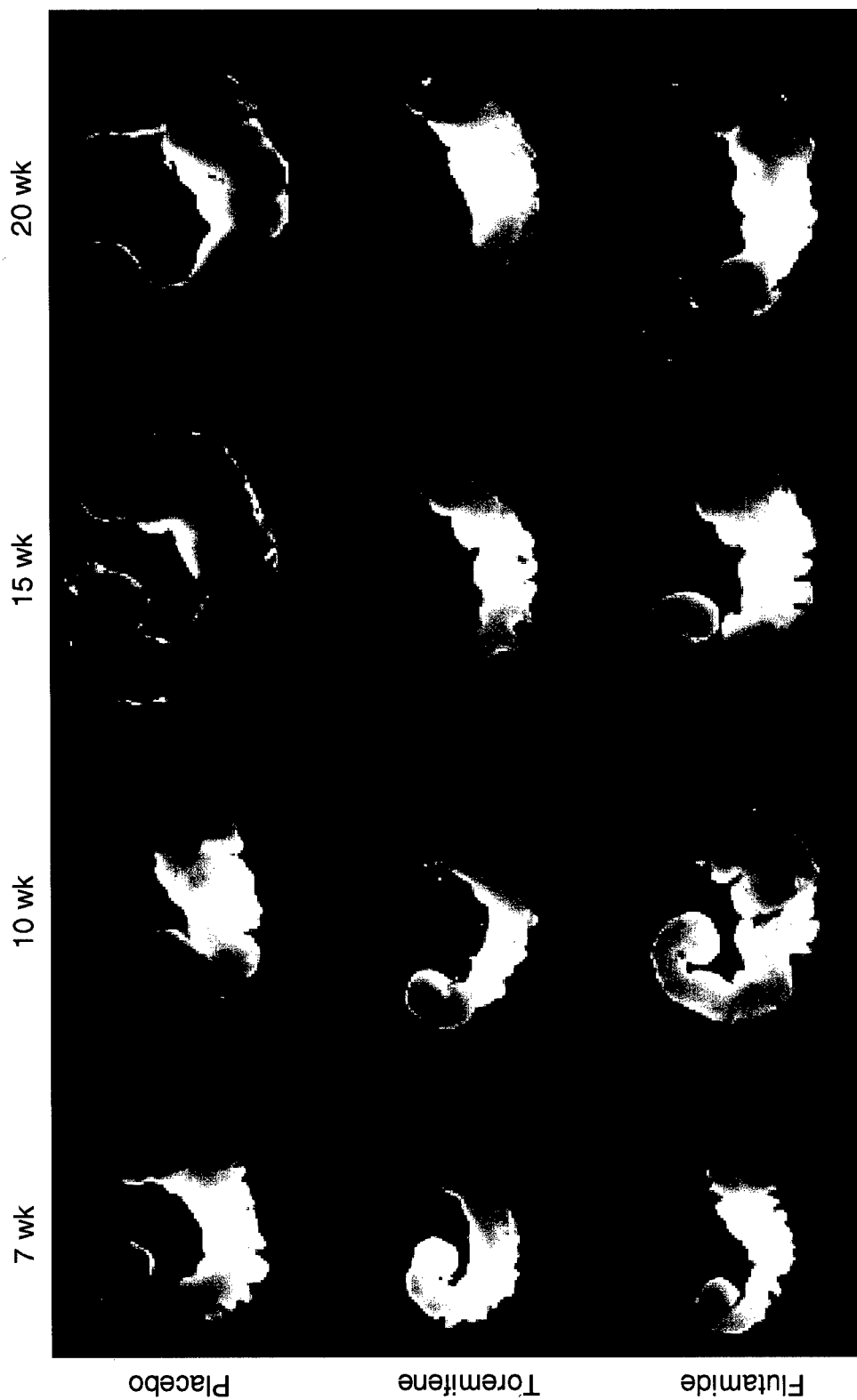


Fig. 2. Effect of Flutamide and Toremifene vs. the Placebo on Seminal Vesicle Development in the TRAMP mouse.

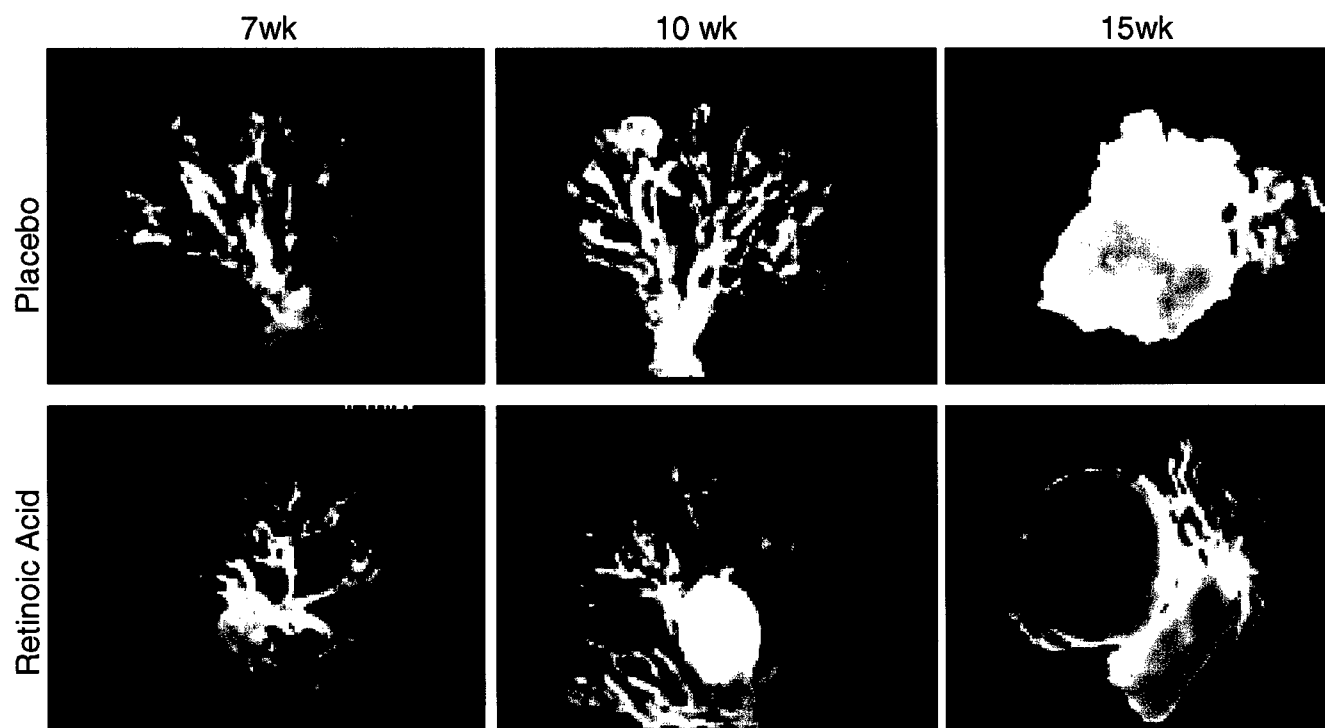


Fig. 3. Effect of Retinoic acid vs. the Placebo on the Ventral Prostate development in the TRAMP mouse.

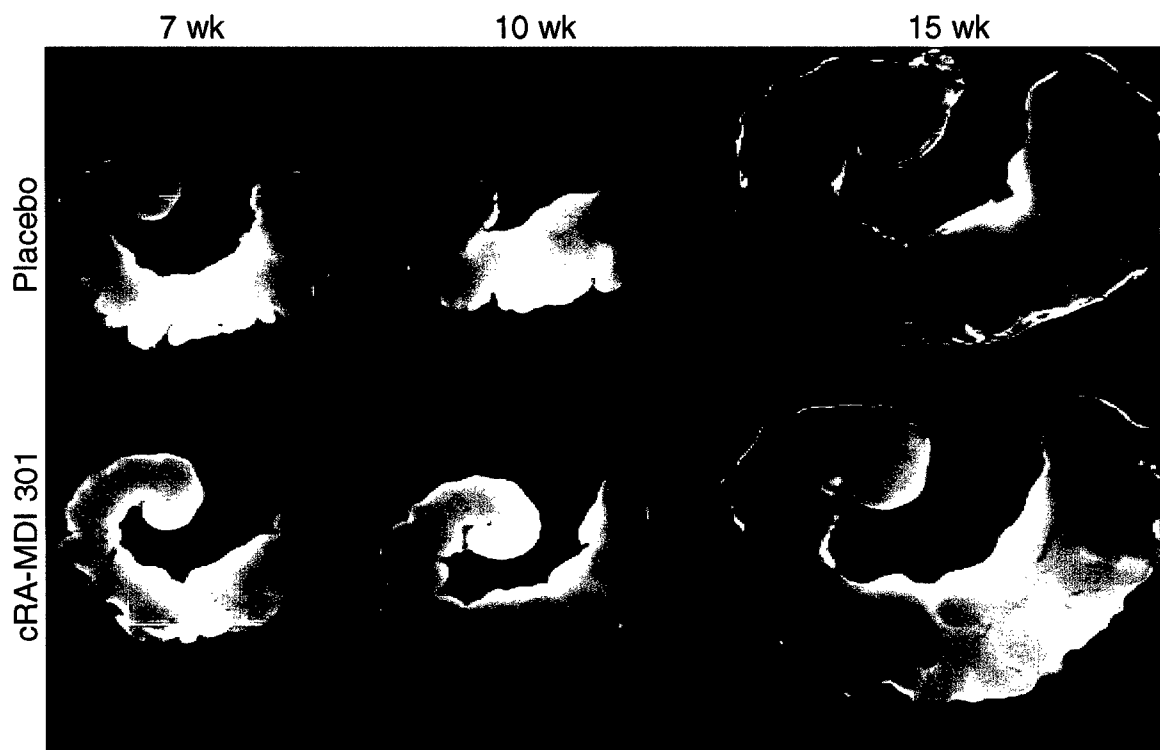


Fig. 4. Effect of cRA MDI 301 vs. the Placebo on Seminal Vesicle Development in the TRAMP mouse.

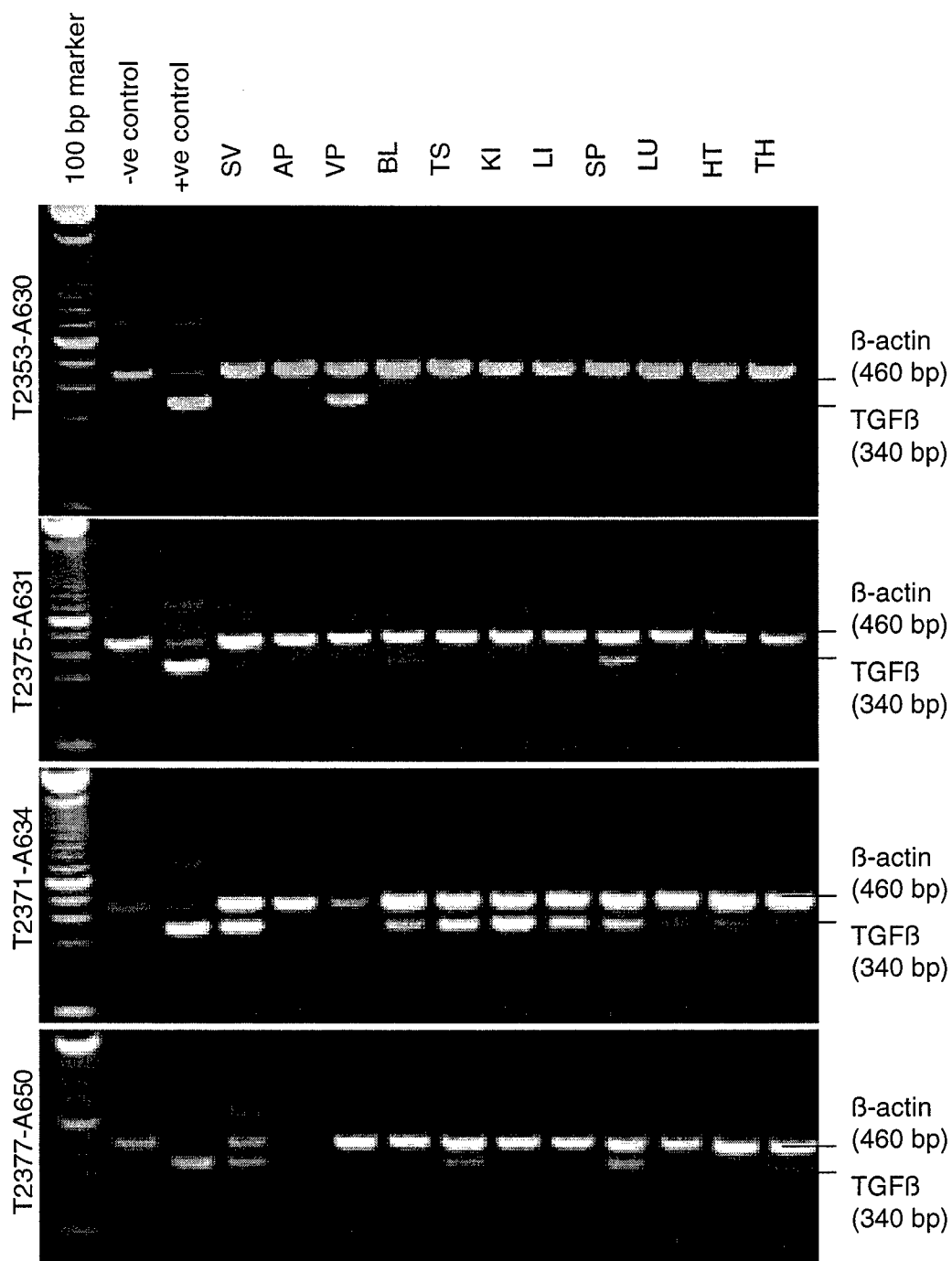


Fig. 5. RT-PCR analyses showing differential TGFβ expression in tissues of transgene-positive pups A630, A631, A634 and A650 from Founder PBTGFβ mice Lines T2353, T2375, T2371 and 2377, respectively. SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus. PCR products: 340 bp TGFβ and 460 bp; β-actin (internal control); -ve control, FVB wild-type tissue; +ve control, MMTVTGFβ mouse tissue.

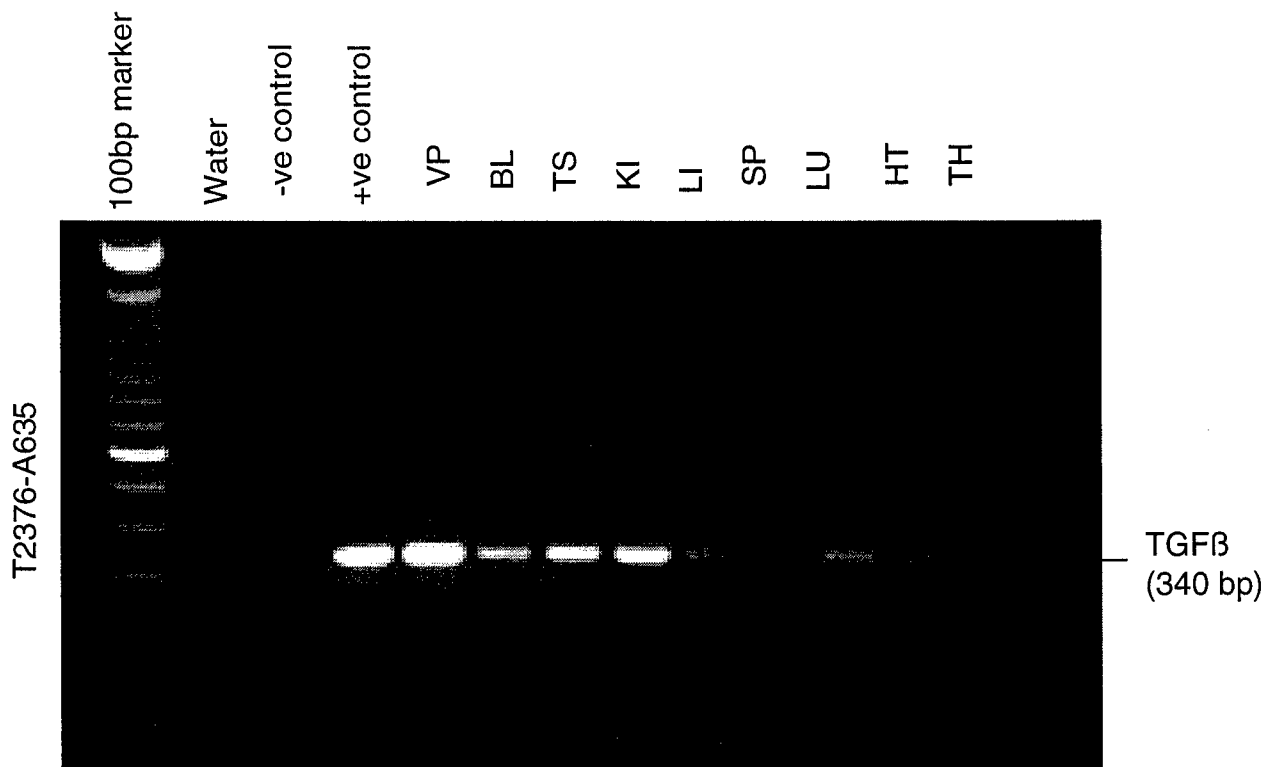


Fig. 6. RT-PCR analyses showing differential TGFβ expression in tissues of transgene-positive pup A635 from Founder mice PBTGFβ-Line T2376. VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus. PCR product 340 bp TGFβ, -ve controls, water and FVB wild-type tissue, +ve control, MMTVTGFβ mouse tissue. Note: Seminal vesicles and anterior prostate did not develop at all in the progeny of this Founder.

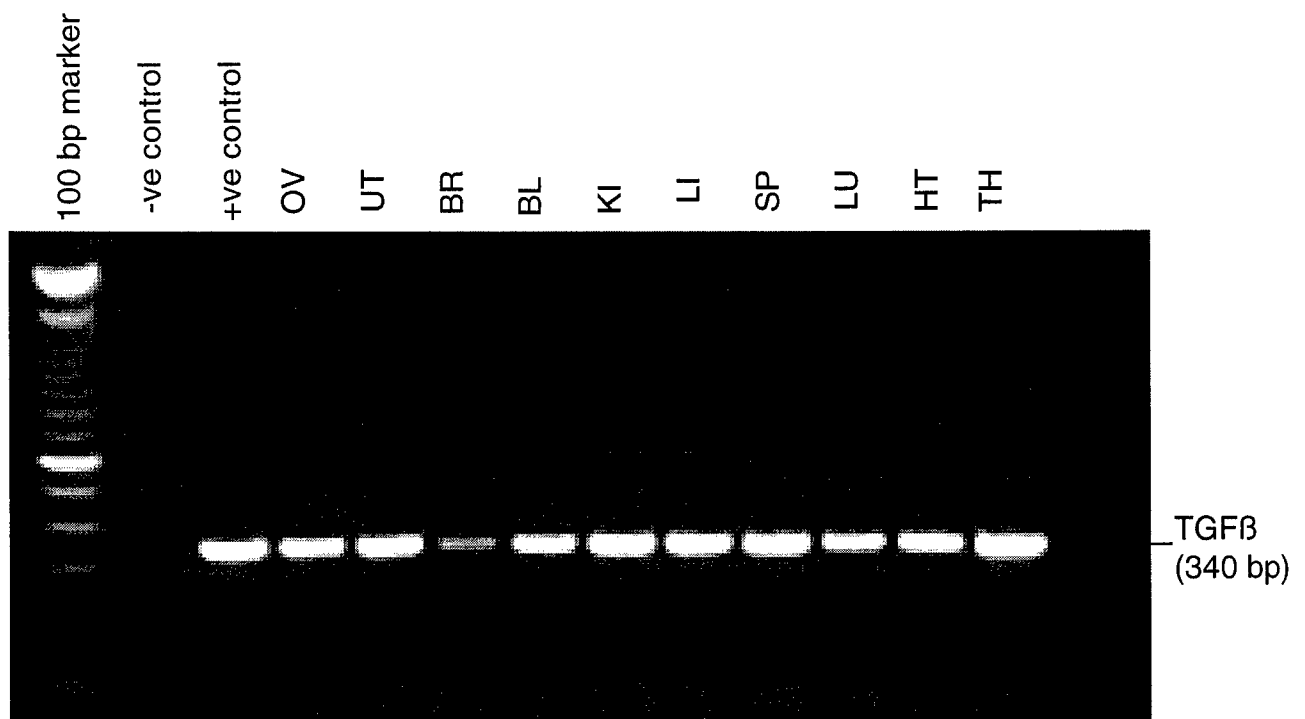


Fig.7. RT-PCR analyses showing differential TGF β expression in tissues of transgene-positive female pup A642 from Founder mice PBTGF β -Line T 2371. OV, ovary; UT, uterus; BR, breast; BL, bladder; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus. PCR products: 340 bp TGF β and 460 bp β -actin (internal control), -ve control, FVB wild-type tissue, +ve control, MMTVTGF β mouse tissue.

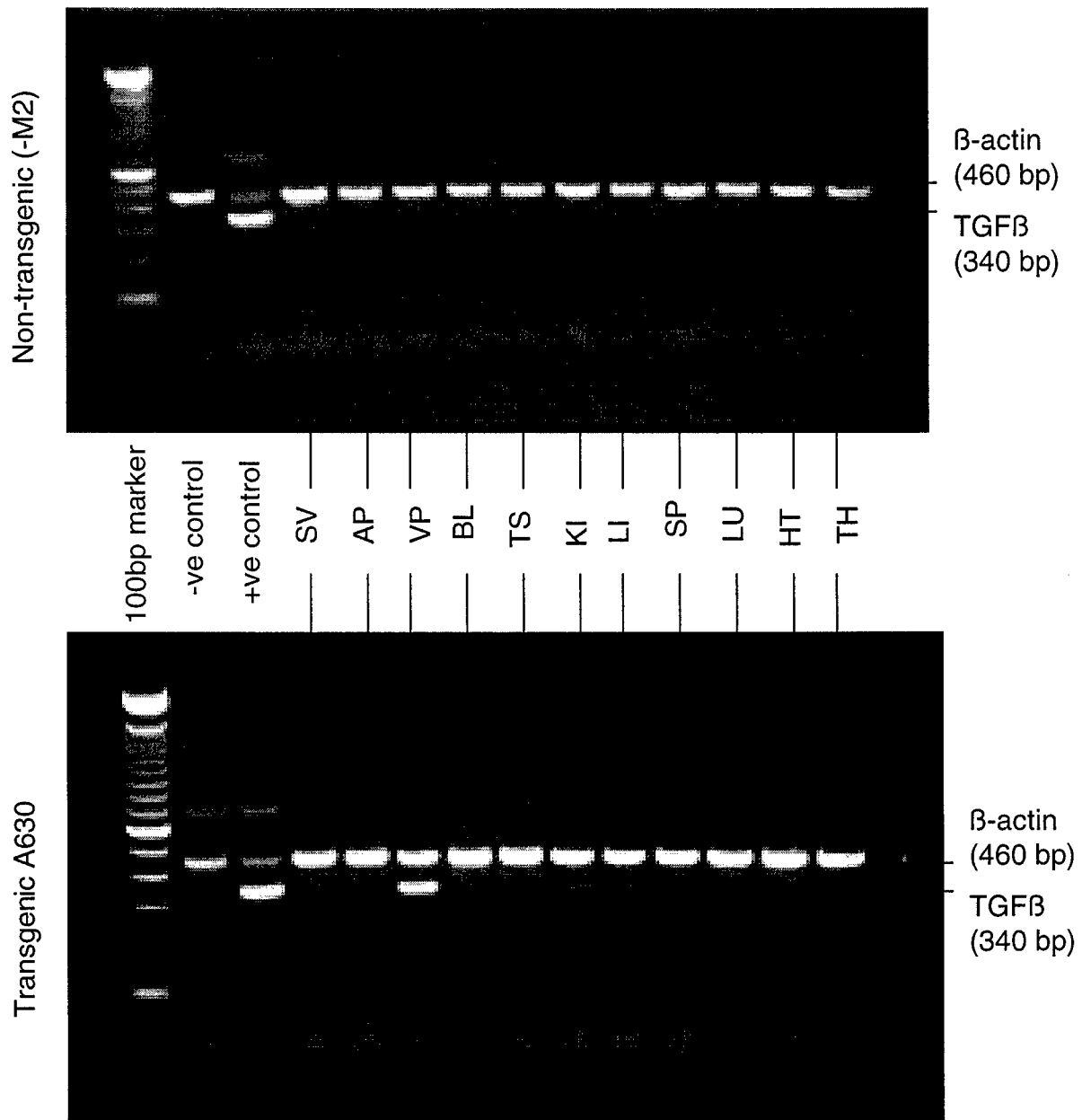


Fig. 8. Expression of the PBTGFβ gene construct in the non transgenic (-M2) vs. transgenic (A630) progeny of the Founder T2353. Tissue distribution of the TGFβ expression was analyzed by RT-PCR, yielding the 340 bp TGFβ and 460 bp β-actin (internal control) PCR products. PCR -ve control, FVB wild-type tissue; +ve control, MMTVTGFβ transgenic mouse tissue. SV, seminal vesicle; AP, anterior prostate; VP, ventral prostate; BL, bladder; TS, testes; KI, kidney; LI, liver; SP, spleen; LU, lung; HT, heart; TH, thymus.

Table 2. TGF β transgene expression by RT-PCR in tissues of PBTGF β mice Founder Lines T2353, T2371, T2375, T2376 and T2377.

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

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; '+', TGF β present; '-', no TGF β ; 0, organ not present.

F1, F1 generation; NT 2-M, nontransgenic male; NT 20-F, nontransgenic female.

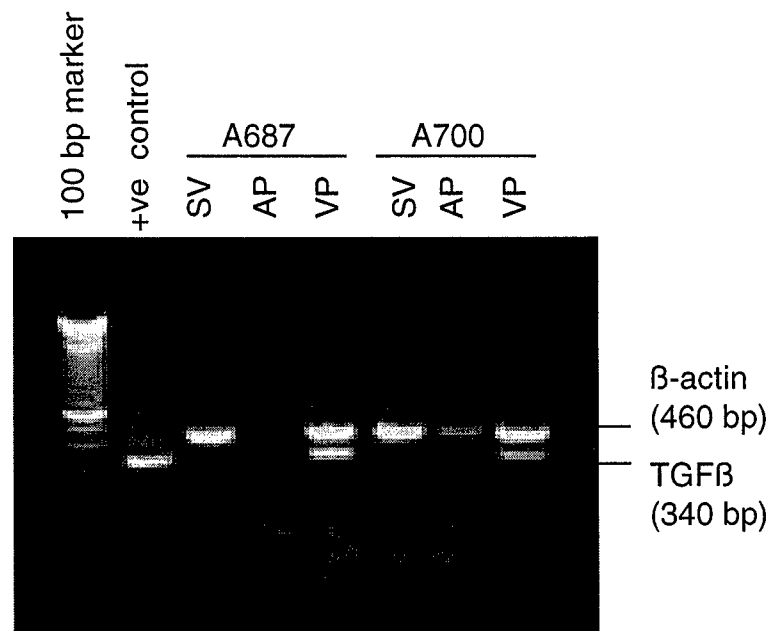


Fig. 9. RT-PCR analyses of tissues from F2 generation pups, A687, A700 of Founder Line T2353 to confirm TGF β expression. SV, seminal vesicle ; AP, anterior prostate; VP, ventral prostate. PCR products: 340 bp TGF β and 460 bp β -actin (internal control); -ve control, FVB wild-type tissue; +ve control, MMTVTGF β mouse tissue.

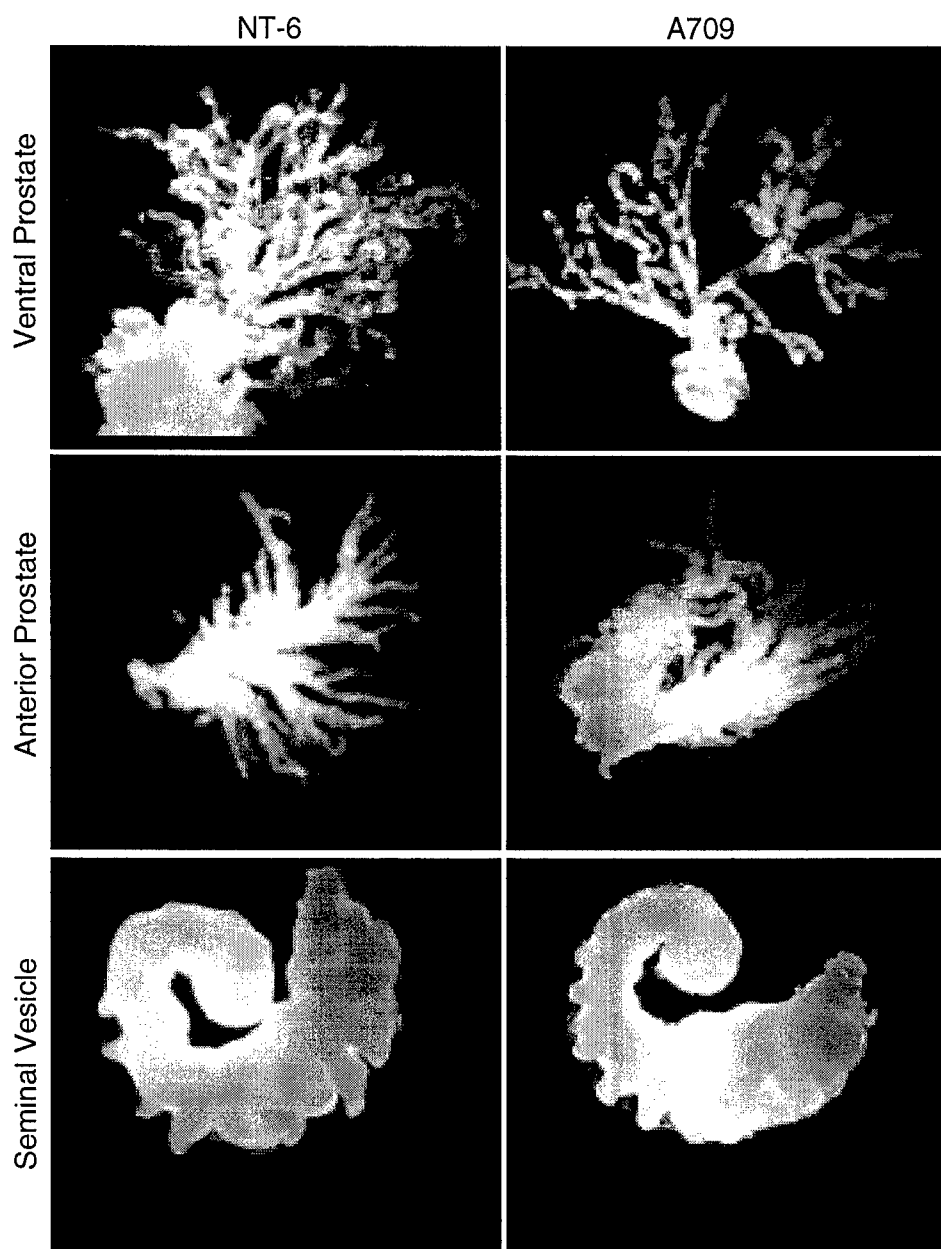


Fig. 10. Dark-field microscopy showing wholemount analyses of Ventral Prostate, Anterior Prostate and Seminal Vesicle from A709 (transgenic PBTGF β) vs. NT6 (non-transgenic) mouse at 7 weeks age.

APPENDICES

1. Sharan Raghow, Emin Kuliyeu, Marjorie Steakley, Norman Greenberg, and Mitchell S. Steiner. **Efficacious chemoprevention of primary prostate cancer by flutamide in an autochthonous transgenic model.** Submitted to Cancer Research September 1999.
2. Raghow et al. **Immunohistochemical localization of transforming growth factor- α and transforming growth factor β during early human fetal prostate development.** Journal of Urology, 1999; 162: 509-513.

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

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Running Title: Flutamide as a chemopreventive agent

**Key Words: prostate cancer, chemoprevention, antiandrogen, TRAMP mice, p53
and pRb**

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ABSTRACT

One third of men over 50 years of age have a latent form of prostate cancer that may develop into a potentially life-threatening clinical prostate cancer. Environmental factors have been shown to play a role in activating latent prostate cancer. A pilot study was undertaken to test the efficacy of Flutamide (anti-androgen) in the TRAMP transgenic model. Three groups of mice received subcutaneous implantations of slow release Flutamide pellets: low dose (6.6 mg/kg), high dose (33 mg/kg), and control placebo animals. Efficacy was measured by the absence of palpable tumor formation. Prostate tissues/tumors were harvested for evaluation by molecular and histologic techniques. The low dose Flutamide group (n=12) was not significantly different than the placebo group (n=10) where palpable tumors initially presented at 17 weeks of age and by 33 weeks all 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 in the high dose Flutamide group was 33 weeks, the low dose Flutamide group was 24.5 weeks, and the placebo was 24.5 weeks. The difference between the placebo and the high dose Flutamide groups was statistically significant (Log rank, $p=0.0036$ and Wilcoxon's statistical analysis, $p=0.0060$). Tumors from high dose Flutamide-treated animals were more differentiated retaining much of the normal glandular architecture compared to the placebo group whose tumors consisted of sheets of poorly differentiated cells. The expression of T antigen was significantly downregulated in the prostate tissues at 10 weeks age in the Flutamide-treated animals. Flutamide had the ability to suppress T-antigen driven carcinogenesis resulting in a significant decrease in the incidence of and 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 for which there is no cure and the prognosis is dismal. One approach may be to find prostate cancer earlier through screening programs to reduce the number of advanced prostate cancer patients. Another strategy is to develop drugs that may prevent prostate cancer. One third of all men over 50 years of age have a latent form of prostate cancer that may be activated into the life-threatening, clinical prostate cancer form. 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). The number of people with latent prostate cancer is the same across all cultures, ethnic groups, and races, yet the frequency of clinically aggressive cancer is markedly different. This suggests that environmental factors may play a role in activating latent prostate cancer. Thus, the development of chemoprevention strategies against prostate cancer may indeed be plausible, and perhaps, have the greatest overall medical and economical impact against prostate cancer.

Prostate cancer, like other cancers, is a multi-step process that results from the accumulation of genetic mutations. These mutations lead prostate cells to acquire both increased sensitivity to positive growth factors or over-expression of oncogenes and/or decreased responsiveness to growth inhibition by negative growth regulators and loss of tumor suppressor genes; the net effect is unregulated cellular proliferation. Evidence suggests that modifications in environmental, dietary, endocrine, or genetic factors may help prevent prostate cancer by interfering with this process. The fact that prostate cancer is initially androgen dependent and that eunuchs do not develop prostate cancer suggest that androgen deprivation may be one way to prevent carcinogenesis. Approaches to influence tissue androgen levels include: 1) Inhibit the pituitary secretion of LH by LHRH analogues; 2) Prevent the conversion of testosterone (T) to dihydrotestosterone (DHT) by 5α -reductase in the prostate; and, 3) to block the prostatic androgen receptors by steroid-like antagonists with no intrinsic activity to reduce the potentially unacceptable

systemic toxicity. One such agent may be the non-steroidal anti-androgen Flutamide that exerts its effects by directly binding to the androgen receptor and interfering with androgen receptor binding of DHT or T (3). Unfortunately, progress in the study of chemoprevention of prostate cancer has been impeded by lack of reliable animal models of human prostate cancer. Recently, a unique animal model known as the transgenic adenocarcinoma of the mouse prostate (TRAMP) of prostate cancer has been described (4). In these mice the prostate specific promoter Probasin (PB), directs expression of the T antigen (Tag) exclusively in prostate cells. Consequently, these mice develop progressive forms of prostate cancer by 17 weeks of age with 100% frequency (5). Moreover, these animals eventually develop metastatic prostate cancer that spreads in a pattern similar to that observed in human prostate cancer (5). Thus, the TRAMP model of prostate cancer represents the first reliable animal model of spontaneous prostate cancer. The ability to identify animals predestined to develop prostate cancer and to modify their environment by chemoprevention 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. Herein, 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 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 the National Institute of Health guidelines for proper and humane use of animals. The PB-Tag transgenic C57BL/6 strain of mice was crossbred with FVB wild type strain, the hybrid litters were screened by PCR (4) for presence of the PB-Tag transgene and only the males that screened positive were used in the study. Flutamide powder was made into slow release pellets (Innovative Research of America, Sarasota, FL) and the drug dose was adjusted for growth related changes in weight. The pellets were implanted subcutaneously through a 1 cm incision on the flank into PB-Tag mice (30 days of age; average weight 14 g) anesthetized with metofane (Mallinckrodt, Mundelein, IL). Three groups of 10-12 animals each received a 90 day-release drug pellet of either a low dose Flutamide (6.6 mg/kg), or a high dose Flutamide (33 mg/kg), or a placebo (pellet with no pharmacological activity). Each treated animal received supplemental dosages at 90 days intervals until tumors were palpable. The efficacy of the treatment was measured by the absence of a palpable tumor. Starting at age 10 weeks animals were evaluated weekly for the presence of a palpable tumor, the endpoint of the study. 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 exact test and Wilcoxon's rank test (18). All p values were two-sided.

Wholemounds and Histology

Ventral prostate lobes from representative animals in the placebo-treated and high 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, and processed in Shandon-Lipshaw tissue processor and paraffin-embedded. Tissue sections (4 μ M thick), were stained with hematoxylin and eosin (H&E) for histological evaluation.

Western Blot Analyses

Crossbred T-antigen positive male pups, 5 each, were treated with either placebo or Flutamide pellets at 4 weeks age. Prostate tissues (dorsolateral and ventral lobes) were harvested at 10 weeks age, snap-frozen in liquid N₂ and stored at -80° C. Tissue lysates were prepared using RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris, pH 7.5) containing a cocktail of protease inhibitors (Pefabloc, aprotinin, bestatin, leupeptin and pepstain) and the phosphatase inhibitor Na₃VO₄ (10mM). The homogenate was centrifuged at 14,000x g at 4° C for 10 minutes and lysates stored at -80° C until used.

Protein concentrations were determined by the Bradford protein assay (Bio-Rad). Tissue lysates were loaded onto 7.5% polyacrylamide gels, proteins (40µg/lane) separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (0.2 µm, Bio-Rad, Hercules, CA) using a transfer buffer (192 mM glycine, 25 mM Tris-Hcl and 20% methanol). TRAMP prostate tumor tissue was used as positive control. Chemiluminescent Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA) were used as MW standards. Blots were blocked overnight at 4°C in BLOTTO (6% non-fat dry milk in 1X TBS) and incubated with the large T-antigen primary antibody (Pab 101 mouse monoclonal, 1:200, Santa Cruz Biotechnology) for 2 hours at room temperature. The blots were washed (3x) with TTBS (0.05% Tween 20, 50mM Tris-Hcl, 200mM NaCl) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1 hour at 25° C. Immunoreactive proteins were visualized on autoradiography film using the enhanced chemiluminescence (ECL) system (APB, Piscataway, NJ). Actin protein expression was used to normalize T-antigen results. For this purpose, the above membrane was submerged in stripping buffer (100 mM 2-mercaptoethanol, 2%SDS, 62.5mM Tris-Hcl pH 6.7) and incubated at 50° C for 30 minutes with occasional agitation. After blocking the membrane was reprobed with Actin primary antibody (1:2500, Chemicon, Temecula, CA) followed by (HRP)-conjugated secondary antibody (1:10000). Following ECL detection, band intensities were quantitated using 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 (n=12) and the placebo (n=10) groups. In both these groups, tumors initially presented at 17 weeks of age, and by 33 weeks of age, all the animals had developed palpable tumors. In the high dose Flutamide 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 (Figure 1). The period of time at which 50% of the animals had palpable tumors in the high dose Flutamide group was 33 weeks of age, the low dose Flutamide group was 24.5 weeks of age, and the placebo group was 24.5 weeks of age. The difference between the placebo and the high dose Flutamide groups was statistically significant both by both log rank and Wilcoxon analysis at a p value 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 wholemount analysis of prostate tissue of representative animals from the placebo-treated and the high Flutamide-treated groups (Figures 2, A-D and E-H, respectively). Tumor mass of fused ducts was visible as early as 15 weeks (Figure 2C) in the placebo group whereas the ducts remained distinct and clear in the Flutamide-treated group as seen at 15 and 20 weeks (Figures 2 G&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 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 (Figure 3). 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 (Figure 4A) was replaced by sheets of undifferentiated, anaplastic cells with a high mitotic index (Figure 4B). Tumors from the low dose Flutamide-

treated (Figure 4C) group were not different from the placebo group. In contrast, the high dose Flutamide-treated (Figure 4D) mice had tumors that were distinctively differentiated retaining a glandular architecture. The mitotic index was much lower than the placebo group. Thus, Flutamide treatment significantly decreased the incidence of, and increased the latency period of prostate cancer in TRAMP mice. Moreover, mice treated with high dose Flutamide had more differentiated tumors.

T antigen was present in the prostate tumor tissue resected at 24 weeks age. The oncoprotein was also present in the placebo-treated 10-week-old animals as confirmed by Western blotting. On the other hand Flutamide-treated animals expressed significantly lower levels of the T antigen than the comparable placebo-treated animals (Figure 5).

DISCUSSION

Cancer is a consequence of a series of spontaneous genetic mutations that accumulate over time to produce a malignant cell. As rapidly proliferating cells appear to be more susceptible to mutations, it is not surprising that the presence of stimulatory growth factors such as testosterone may also contribute to carcinogenesis (6,7). Hormonal factors appear to be important for development of prostate cancer as eunuchs do not have prostate cancer, and prostate cancer can be induced in Noble rats by the chronic administration of testosterone (8,9). Androgens regulate prostatic epithelial proliferation by modulating stimulatory and inhibitory growth factors to maintain homeostasis. As androgen promotes carcinogenesis, its inhibition remains a logical first approach for prostate cancer prevention. Gingrich et al. (1997) examined the consequences of androgen deprivation by castration on the initiation and progression to metastatic prostate cancer in TRAMP mice. Their studies revealed that although castration at 12 weeks 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. (11) reported a marked decrease in the prevalence and extent of high grade intraepithelial neoplasia in prostates of patients receiving androgen-deprivation therapy compared with prostates of untreated patients. Finasteride, a 5 α -reductase inhibitor, is currently being investigated as an agent to prevent prostate cancer, in the NCI-sponsored Prostate Cancer Prevention Trial. However, its ability to prevent prostate cancer in animals has never been demonstrated. Consequently, other agents with demonstrable efficacy against prostate cancer oncogenesis should be explored.

The study of prostate cancer chemoprevention has been hindered by the lack of appropriate animal models. The recent development of the transgenic adenocarcinoma mouse prostate (TRAMP) model has allowed for the first time a means to effectively study chemoprevention. In the TRAMP model (4) the PB-Tag transgene is expressed specifically in the epithelial cells of the murine prostate. As a result, this model has several advantages over the currently existing models: 1) the mice develop prostatic epithelial hyperplasia and prostatic intraepithelial neoplasia, a premalignant lesion, as early as 10 weeks and invasive adenocarcinoma around 18 weeks of age,

2) the pattern of prostate cancer metastasis mimics that of human prostate cancer, 3) the development as well as the progression of prostate cancer can be followed within a relatively short period of 10-30 weeks, 4) the tumors occur with 100% frequency, and 5) the animals may be screened for the presence of the prostate cancer transgene prior to the onset of clinical prostate cancer to directly test whether treatment with chemopreventive agents may alter prostate carcinogenesis.

This present study also used a better drug delivery method than the previous prostate cancer chemoprevention studies. (12). The slow-release subcutaneous-implanted pellets provide a more controlled and more reliable drug dosage than the conventionally used ad libitum diet method that may introduce significant variability. Using this approach, the high dose Flutamide treatment increased the latency period of prostate cancer by 7 weeks. Thus, the disease was significantly ($7/24=29\%$) delayed and the tumors were more differentiated in the 42% of the mice that ultimately developed prostate cancer. These results are in direct contrast to the castration data by Gingrich et al. (1997) where 65% of the castrated animals developed tumors of which 100% were poorly differentiated and suggest that blocking ligand binding is less drastic and probably a better method of androgen deprivation than castration. Also, this implies that castration has broader effects on changing the total environment than Flutamide, that is, castration probably sets up an environment more selective for aggressive/metastatic androgen-independent disease. In addition to the notable delay, the significant decrease in prostate cancer suggests that Flutamide at a higher dose may be an effective chemopreventive agent. Earlier experiments in rats had calculated the minimum effective anti-androgen dose for Flutamide to be 5mg/kg body weight per day (3). Later studies on rats, dogs and baboons used Flutamide at 50 mg/day which was 10 times the minimum effective dose (3, 13). Since the low dose of Flutamide (6.6 mg/day) was totally ineffective in the delay or prevention of prostate cancer in the TRAMP mice, we postulate that a more complete androgen blockade was necessary to elicit its chemopreventive effect. Simard et al. (14) studied the interaction of Flutamide with the androgen receptor in target tissues including the rat ventral prostate and human prostatic carcinoma. Based on the relative affinities of DHT, T and FLU-OH

(hydroxy-Flutamide, the active metabolite of Flutamide), higher concentration anti-androgens were needed to efficiently prevent androgen receptor binding by androgen (15).

Flutamide exerts its anti-androgen influence by blocking ligand binding to the androgen receptor (3). It appears that in the TRAMP model this anti-androgen influence is conferred upon, and results in, the decreased expression of the T-antigen through the androgen responsive elements of the PB promoter. This, in turn, relieves more of the p53 protein for its assigned role as the cellular gatekeeper of growth and division (16) resulting in delay of prostate cancer. The SV40 large T antigen has been shown to bind and inactivate the p53 tumor suppressor gene product (17) and the loss of tumor-suppressor wild-type p53 and Rb genes has been implicated in development of prostate cancer (18, 19). Since, androgens promote carcinogenesis and androgen deprivation is still the choice therapy prostate cancer, the TRAMP model with its hormone-responsive PB promoter behaves predictably and, thus, presents a valid model to assess the efficacy of potential androgen analogs. However, it may be a suitable model to study molecular mechanisms of carcinogenesis and the efficacy of other chemopreventive agents in the event the agent causes delay or prevention of cancer without affecting the T-antigen levels in the course of treatment.

Histological examination showed that tumors from Flutamide-treated animals were more differentiated, retaining much of the glandular architecture compared to the placebo group suggesting that Flutamide was able to interfere with tumor differentiation. Interestingly, overexpression of TGF β 1, an inhibitor and differentiation factor, has been shown to reduce breast cancer tumor formation in transgenic mice raising the possibility that TGF β stimulating agents may also prevent other hormone responsive tumors like prostate cancer (15, 20-22). Flutamide also has been shown to stimulate TGF β production in regressed human prostate cancer (23) and induces involution of rat normal prostate (24). This suggests that chemopreventive effects of Flutamide might involve TGF β 1 inducing differentiation mechanism.

At the effective high dose used in our study, Flutamide was well tolerated in these animals with no obvious signs of toxicity. The toxicity profile of Flutamide, unlike retinoic acids, in human studies is reportedly favorable (25). Moreover, since Flutamide works at the prostate level

and consequently, testosterone blood levels are not reduced, libido and potency are maintained (26). This is critical because men that currently do not have prostate cancer will only be interested in taking chemopreventive agents that have either no or a very low toxicity profile. Based on these studies, Flutamide should be considered as a test agent in clinical prostate cancer chemoprevention trials.

FIGURE LEGEND

Figure 1. Chemopreventive effects of Flutamide in the TRAMP model. Transgenic mice were divided into three groups: Placebo, Low dose Flutamide (6.6 mg/kg/d) and High dose Flutamide (33 mg/kg/d). Starting at 10 weeks of age, animals were examined weekly for the presence of a palpable tumor. Each point represents number of animals without palpable tumors (% tumor-free) in the Kaplan-Meier graph.

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

Figure 3. Effect of Flutamide on prostate tumor development in the TRAMP model. (H&E) stain, magnification 66X.

Figure 4. Histology of tumors from the placebo and Flutamide treated transgenic mice. Representative H&E stained tissue sections from the normal murine prostate (4A; magnification 66X) and various tumors in treated transgenic mice are shown. Both placebo treated tumors (4B; magnification 66X) and low Flutamide treated tumors (4C; magnification 132X) were composed of poorly differentiated sheets of malignant cells with no recognizable original glandular prostate architecture. In contrast, the high Flutamide treated tumors (4D; magnification 132X) was more differentiated and retained more of the original glandular architecture.

Figure 5. Effect of Flutamide-treatment upon T-ag expression in the TRAMP mouse prostate. Western Blot using prostate tissue lysates (40µg protein/lane) of 10 week-old placebo- or Flutamide-treated mice. (A) top, probed with anti-large T-antigen mouse monoclonal IgG and bottom, reprobed with anti-actin mouse monoclonal IgG as internal control. (B) Densitometric volume of the T-antigen and actin bands.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., Wingo, P. A. Cancer Statistics 1998. *CA Cancer J Clin*, 48: 6-29, 1998.
2. Sheldon, C. A., Williams, R. D., and Fraley, E. E. Incidental carcinoma of the prostate: A review of the literature and critical reappraisal of classification. *J. Urol.*, 124: 626-631, 1980.
3. Neri, R. Pharmacology and pharmacokinetics of Flutamide. *Urology*, 34 (4 Suppl): 19-21, 1989.
4. Greenberg, N. M., DeMayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O., Cunha, G.R., Donjacour, A. A., Matusik, R. J., and Rosen, J. M. Prostate cancer in a transgenic mouse. *Proc. Natl. Acad. Sci.*, 92: 3439-3443, 1995.
5. Gingrich, J. R., Barrios, R. J., Morton, R. A., Boyce, B. F., DeMayo, F. J., Finegold, M. J., Angelopoulou, R., Rosen, J. M., and Greenberg, N. M. Metastatic prostate cancer in a transgenic mouse. *Cancer Res.*, 56: 4096-4102, 1996.
6. Walsh, P. C. Prostate cancer kills: Strategy to reduce deaths. *Urology*, 44: 463-466, 1994.
7. Steiner, M. S. The role of peptide growth factors in the prostate: A review. *Urology*, 42: 99-110, 1993.
8. Noble, R. L. The development of prostatic adenocarcinoma in the Nb rat following prolonged sex hormone administration. *Cancer Res.*, 37: 1929, 1977.
9. Stamey, T. A., and McNeal, J. E. Adenocarcinoma of the prostate. *In*: P.C. Walsh, A. B. Retik, T. A. Stamey, and E. D. Vaughan, Jr. (eds.), *Campbell's Urology*, 6th ed., pp. 1159-1199. Philadelphia: W.B. Saunders, 1992.
10. Gingrich, J. R., Barrios, R. J., Kattan, M. W., Nahm, S. H., Finegold, M. J., Greenberg, N. M. Androgen-independent prostate cancer progression in the TRAMP model. *Cancer Res.*, 57: 4687-4691, 1997.

11. Ferguson, J., Zincke, H., Ellison, E., Bergstralh, E., and Bostwick, D. G. Decrease of prostatic intraepithelial neoplasia following androgen deprivation therapy in patients with stage T3 carcinoma treated by radical prostatectomy. *Urology*, 44: 91-95, 1994.
12. Anzano, M. A., Peer, C. W., Smith, J. M., Mullen, L. T., Shrader, M. W., Logsdon, D. L., Driver, C. L., Brown, C. C., Roberts, A. B., and Sporn, M. B. Chemoprevention of mammary carcinogenesis in the rat: Combined use of raloxifene and 9-cis retinoic acid. *J. Natl. Cancer Inst.*, 88: 123-125, 1996.
13. Husmann, D. A., McPhaul, M. J., and Wilson, J. D. Androgen receptor expression in the developing rat prostate is not altered by castration, Flutamide or suppression of the adrenal axis. *Endocrinology*, 128: 1902-1906, 1991.
14. Simard, J., Luthy, I., Guay, J., Belanger, A., and Labrie, F. Characteristics of interaction of the anti-androgen Flutamide with the androgen receptor in various target tissues. *Molecular and Cellular Endocrinology*, 44: 261-270, 1986.
15. Thompson, T. C. Growth factors and oncogenes in prostate cancer. *Cancer Cells*, 2: 345-354, 1990.
16. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell (United States)*, 88: 323-331, 1997.
17. Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumor suppressor gene. *Nature (England)*, 351: 453-456, 1991.
18. Bookstein, R., Rio, P., Madreperla, S. A., Hong, F., Allred, C., Grizzle, W. E. and Lee, W. H. Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. *Proc. Natl. Acad. Sci. USA*, 87: 7762-7766, 1990.
19. Rubin, S. J., Hallahan, D. E., Ashman, C. R., Brachman, D. G., Beckett, M. A., Virudachalam, S., Yandell, D. W., Weichselbaum, R. R. Two prostate carcinoma cell lines demonstrate abnormalities in tumor suppressor genes. *J. Surg. Oncol. USA*, 46: 31-36, 1991.

20. Rijnders, A. W. M., van der Korput, J. A. G. M., Van Steenbrugge, G. J., Romijn, J. C., and Trapman, J. Expression of cellular oncogenes in human prostatic carcinoma cell lines. *Biochem. Biophys. Res. Comm.*, 132: 548-554, 1985.
21. Pierce, D. F., Johnson, M. D., Matsui, Y., Robinson, S. D., Gold, L. I., Puchio, A. F., Daniel, C. W., Hogan, B. L. M., and Moses, H. L. Inhibition of mammary duct development but not of alveolar outgrowth during pregnancy in transgenic mice expressing active TGF β 1. *Genes Dev.*, 7: 2308-2317, 1993.
22. Pierce, Jr., P. F., Gorska, A. E., Chytil, A., Meise, K. S., Page, D. L., Coffey, Jr., R. J., and Moses, H. L. Mammary tumor suppression by transforming growth factor- β 1 transgene expression. *Proc. Natl. Acad. Sci. USA*, 92: 4254-4258, 1995.
23. Knabbe, C., Klein, H., Zugmaier, G., and Voigt, K. D. Hormonal regulation of transforming growth factor- β 2 expression in human prostate cancer. *J. Steroid Biochem. Molec. Biol.*, 4: 137-142, 1993.
24. Neri, R. O., and Peets, E. A. Biological aspects of anti-androgens. *J. Steroid Biochem.*, 6: 815, 1975.
25. Vassilomanolakis, M., Koumakis, G., Barbounis, V., Hajichristou, H., Tsousis, S., and Efremidis, A. A phase II study of Flutamide in ovarian cancer. *Oncology*, 54: 199-202, 1997.
26. Soloway, M. S., and Matzkin, H. Anti-androgenic agents as monotherapy in advanced prostatic carcinoma. *Cancer*, 71(3 Suppl): 1083-1088, 1993.

Figure 1

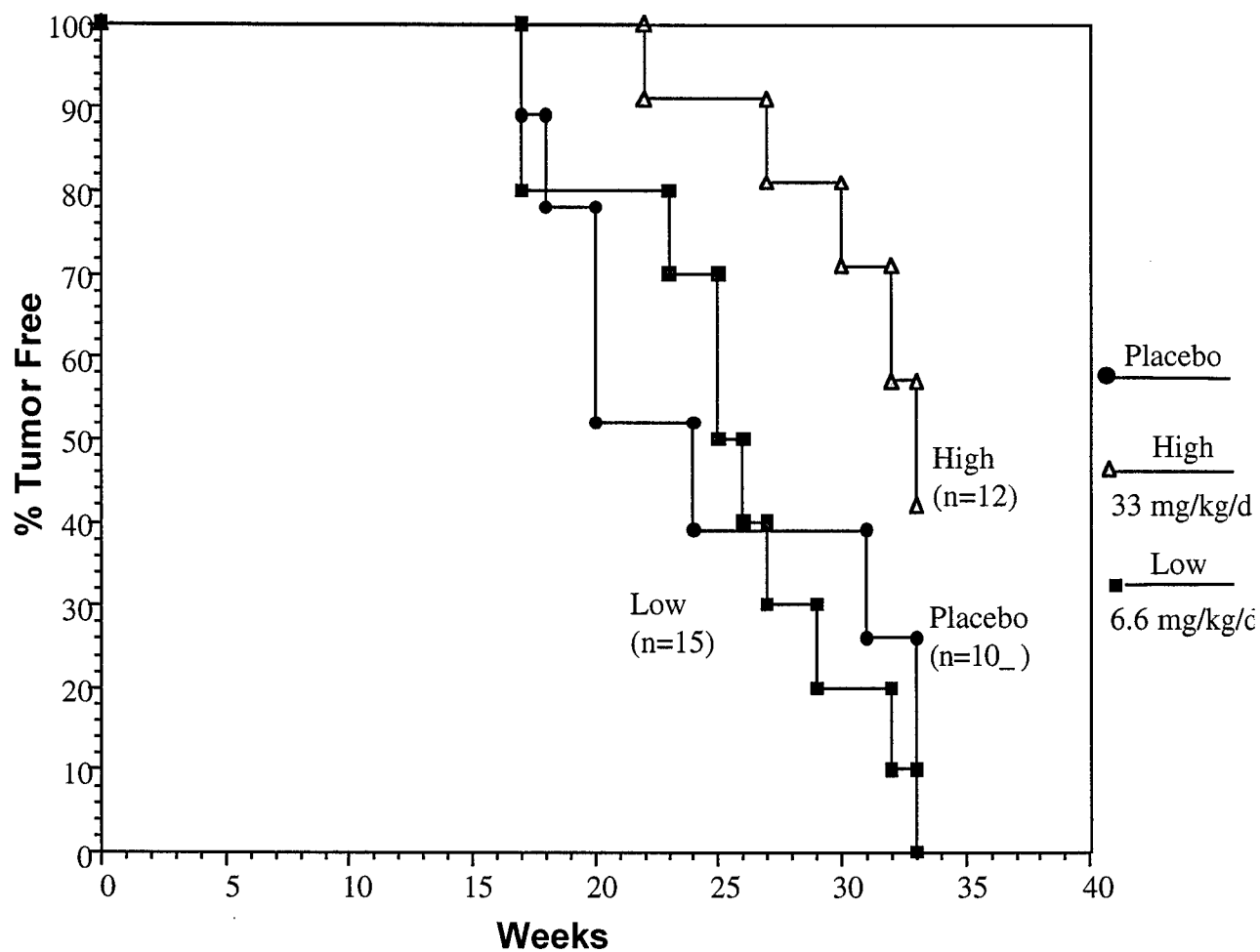


Table 1. Statistical Analysis

	Log-Rank (p value)	Wilcoxon (p value)
Low flutamide vs placebo	0.7955	0.8628
High flutamide vs placebo	0.0036*	0.0060*

* p= < 0.05 level of significance

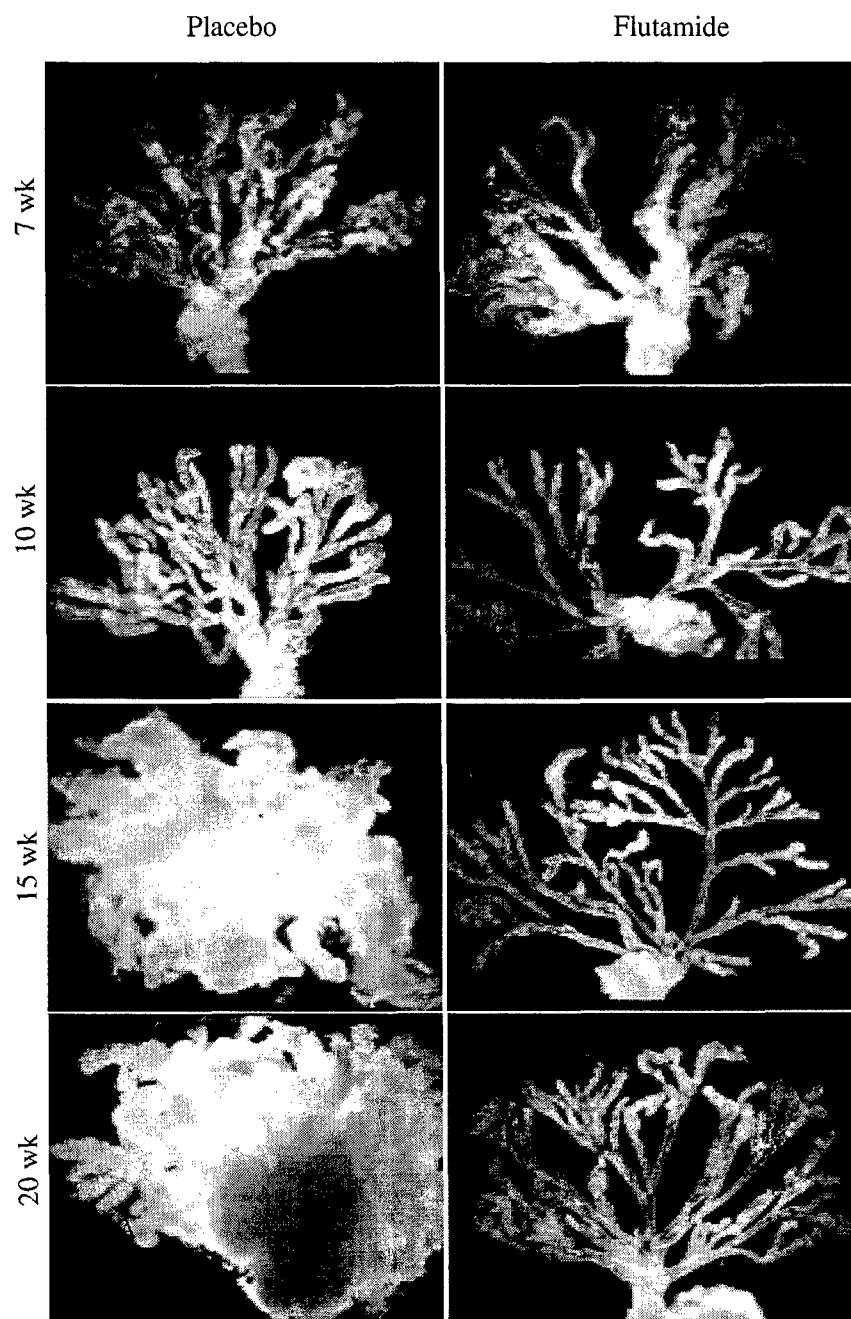
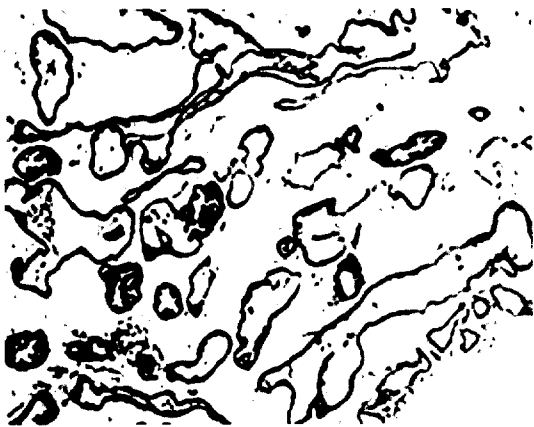
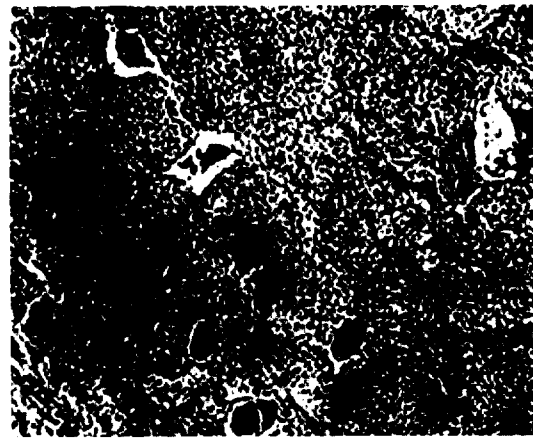


Fig. 2. Effect of Flutamide on prostate tumor development in the TRAMP model. Dark-field microscopy of Ventral prostate whole-mounts showing prostatic ducts joining the urethra. A-D, Placebo-treated; E-H, Flutamide-treated.



Wild-type mouse ventral prostate-17wk



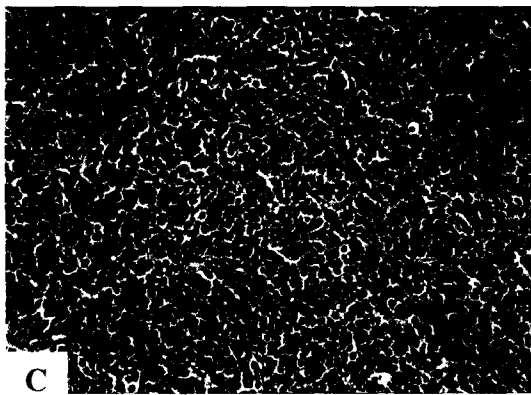
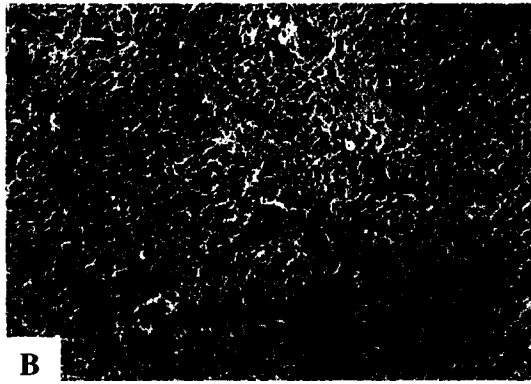
TRAMP ventral prostate-17wk



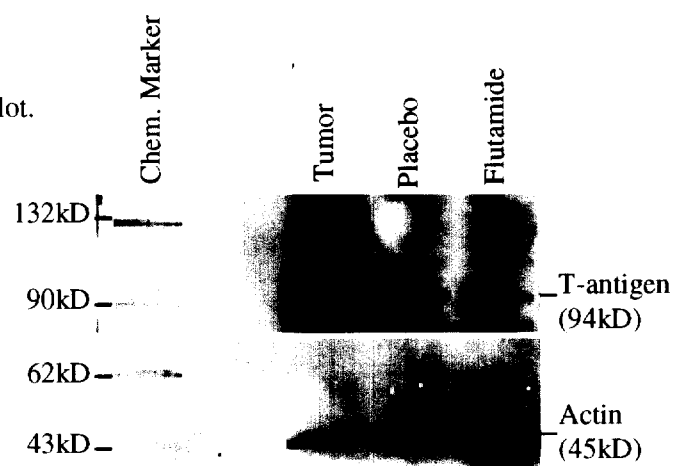
Placebo-treated prostate-15wk



Flutamide-treated prostate-15wk



A. Western Blot.



B. Densitometry.

<u>Sample</u>	<u>T-ag</u>	<u>Actin</u>	<u>T-ag / Actin</u>
Tumor(+ve Control)	69747	12922	5.397539
Placebo	8497	16426	0.517291
Flutamide	6322	25599	0.246963

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

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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- β 1 and TGF- β 3 but not TGF- β 2 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- β 1 decreased and TGF- β 2 increased in the mesenchyme, and TGF- α , TGF- β 1 and TGF- β 3 increased in the epithelium. With declining androgen levels TGF- α , TGF- β 2 and TGF- β 3 remained unchanged but TGF- β 1 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

The human prostate first appears at 9 to 10 weeks of gestation as epithelial buds that form ducts, and begin a 10 to 13-week period of rapid elongation and cellular proliferation. Testosterone produced by the Leydig cells of the fetal testis at about 8 weeks is converted to dihydrotestosterone (DHT) by 5 α -reductase in the prostate, and by 13 weeks further prostate development is dependent on DHT.¹ Under the influence of DHT, the prostatic ducts continue to elongate, canalize and arborize between 13 and 20 weeks of gestation. At this point development of the human fetal prostate proceeds through the bud stage (20 to 30 weeks)—formation of simple solid buds, the bud-tubular stage (31 to 36)—buds tubularize by becoming more cellular and organized, and the acinotubular stage (37 to 42 weeks)—tubules become arranged into lobular clusters.²

Although the precise cellular and molecular mechanisms remain unclear, androgens and peptide growth factors appear to mediate the mesenchymal and epithelial interactions needed for normal prostate morphogenesis. The stroma of the developing prostate, which is thought to be the target of androgen stimulation, elaborates factors that induce glandular proliferation.³ The androgen receptor is expressed prenatally in the mouse prostatic mesenchyme but not in the epithelium.⁴ Based on these observations, Tenniswood proposed that paracrine interactions between the androgen receptor positive mesenchyme and androgen receptor negative epithelium may be mediated by a stromal derived growth factor capable of regulating epithelial cell proliferation.⁵

Prostate organ culture studies have provided direct evidence of such interactions between stroma and epithelium as DHT promotes mitogenesis of stromal cells and stromal cells secrete fibroblast derived soluble growth factors which stim-

ulate epithelial proliferation.⁶ Peptide growth factors appear to be those soluble factors that mediate androgen effects on postnatal prostatic growth but their role in prenatal prostate development is unknown. Nonetheless, experimental evidence examining the interactions of peptide growth factor members of the epidermal growth factor (EGF) and transforming growth factor (TGF)- β families, and androgens in prostate tissue have provided some mechanistic clues. Since EGF is under androgen control and is required for epithelial cell proliferation *in vitro*, it may be one of the stromal derived growth factors.⁷ A member of the EGF family, TGF- α , is preferentially expressed during periods of prenatal and postnatal prostate epithelial development.⁸ TGF- β is a multifunctional family that generally inhibits growth of many types of epithelial cells and stimulates most mesenchymal cells.⁹ In transgenic mice overexpression of TGF- β 1 appears to alter prostate development by decreasing ductal branching and increasing smooth muscle surrounding the acinar ducts.¹⁰ Studies in rats have shown that castration is followed by a cascade of events, including down regulation of TGF- α and a marked increase in TGF- β 1 messenger ribonucleic acid (mRNA) expression and TGF- β 1 receptor binding sites in ventral prostate.¹¹ Finally, other studies have confirmed that some aspects of postnatal prostatic growth are androgen independent, as castration does not completely inhibit prostate development supporting a role for peptide growth factors.¹²

Indirect evidence suggests that it is the interplay between stimulatory growth factors (TGF- α) and inhibitory growth factors (TGF- β 1 to β 3) that regulate in part the mesenchymal and epithelial interactions responsible for prostate development. The exact interrelationship between androgens and peptide growth factors remains to be elucidated. To define the role of peptide growth factors at sequential stages of prostatic growth and development, we examined the spatial

Accepted for publication February 26, 1999.

Supported by National Institutes of Health, National Institute for Diabetes and Digestive and Kidney Diseases Grants R01-47575 and R01-47503-02.

and temporal expression of a mitogenic peptide growth factor TGF- α and the inhibitory growth factors TGF- β 1, TGF- β 2 and TGF- β 3 in early fetal prostate development (9.5 to 20 weeks of gestation). These studies support the hypothesis that peptide growth factors may be the mediators of androgenic action in mesenchymal and epithelial interactions responsible for early prostate development.

MATERIALS AND METHODS

The use of human fetal tissue for this study was approved by the New York University School of Medicine Institutional Review Board. Prostate tissue sections from human fetuses 9.5, 11.5, 13, 16.5, 18 and 20 weeks in gestation was formalin fixed, oriented appropriately and paraffin embedded. The entire prostate glands were serially step sectioned (3 μ M. thick), and 3 sections from the apex, mid-gland (verumontanum) and base were selected for each age group. Immunohistochemistry was performed on 3 μ M. tissue sections that were prewarmed at 60C 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- β 1; 1:100 in 0.5% casein/phosphate buffered saline, polyclonal rabbit antiporcine antibody AB-12 NA for TGF- β 2, and 1:100 in 0.5% casein/phosphate buffered saline, polyclonal goat antichickens antibody AB-244-NA for TGF- β 3). With each experimental run mouse epididymis sections were used as negative (treated with goat or rabbit preimmune serum) and positive (treated with primary antibody) controls. After a thorough cold phosphate buffered saline rinse (5 minutes $\times$ 3 with agitation on a platform shaker), the samples were incubated for 1 hour with the appropriate biotinylated secondary antibody (1:1,000 in phosphate buffered saline, rabbit antigoat IgG for TGF- β 1 and TGF- β 3, and goat antirabbit for TGF- α and TGF- β 2). 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- β 1 and TGF- β 3 were present in the mesenchyme (fig. 2, A, B and D); whereas staining for mesenchymal TGF- β 2 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 5 α -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 action on androgen receptor positive mesenchyme was associated with increased TGF- β 2 immunostaining which was undetectable before the androgen surge (see table and figs. 3, C versus 2, C). There was no change in mesenchymal TGF- α or TGF- β 3 but TGF- β 1 levels declined during this gestational period (see table). DHT produced in the stroma influenced the epithelium by paracrine pathways and was associated with a distinct increase in TGF- α , TGF- β 1 and TGF- β 3 but little change in TGF- β 2 immunostaining (fig. 3).

Between 18 and 20 weeks of gestation TGF- α , TGF- β 2 and TGF- β 3 staining remained intense in the mesenchyme (fig. 4, A, C and D). In addition, mesenchymal TGF- β 1, which had declined during the androgen surge, again increased in immunostaining intensity (fig. 4, B and table). In the epithelium TGF- α , TGF- β 1 and TGF- β 3 staining was similar to that observed during the androgen surge (see table). Paradoxically, in the mesenchyme TGF- β 1 levels increased with declining androgen levels. The persistence of peptide growth factor levels even with declining androgen levels suggests that peptide growth factors may be the key mediators of continued androgen action during this period of rapid prostate morphogenesis.

DISCUSSION

Growth factors have been implicated in benign and malignant growth as possible autocrine and paracrine mediators of

Immunohistochemical analysis staining intensity of TGF- α , TGF- β 1, TGF- β 2 and TGF- β 3 in human fetal prostate at 9.5, 11.5, 13, 16.5, 18 and 20 weeks of gestation

	Pre-Androgen Surge (testosterone less than 40 ng/100 ml.)		Androgen Surge (testosterone 40–580 ng/100 ml.)		Post-Androgen Surge (testosterone less than 100 ng/100 ml.)	
Wks. gestation	9.5	11.5	13	16.5	18	20
TGF- α :						
Prostate mesenchyme	Moderate	Moderate	Strong	Strong	Intense	Intense
Prostate epithelium	None	Weak	Strong	Strong	Strong	Intense
TGF- β 1:						
Prostate mesenchyme	Strong	Weak	Weak	Weak	Weak	Strong
Prostate epithelium	None	None	None	Weak	Weak	Weak
TGF- β 2:						
Prostate mesenchyme	Weak	Weak	Moderate	Weak	Moderate	Weak
Prostate epithelium	None	Weak	Weak	Weak	Weak	Weak
TGF- β 3:						
Prostate mesenchyme	Moderate	Moderate	Strong	Moderate	Moderate	Strong
Prostate epithelium	None	Weak	Moderate	Moderate	Moderate	Moderate

Slides were scored for reddish-brown intensity of the immunostain by 2 independent investigators.

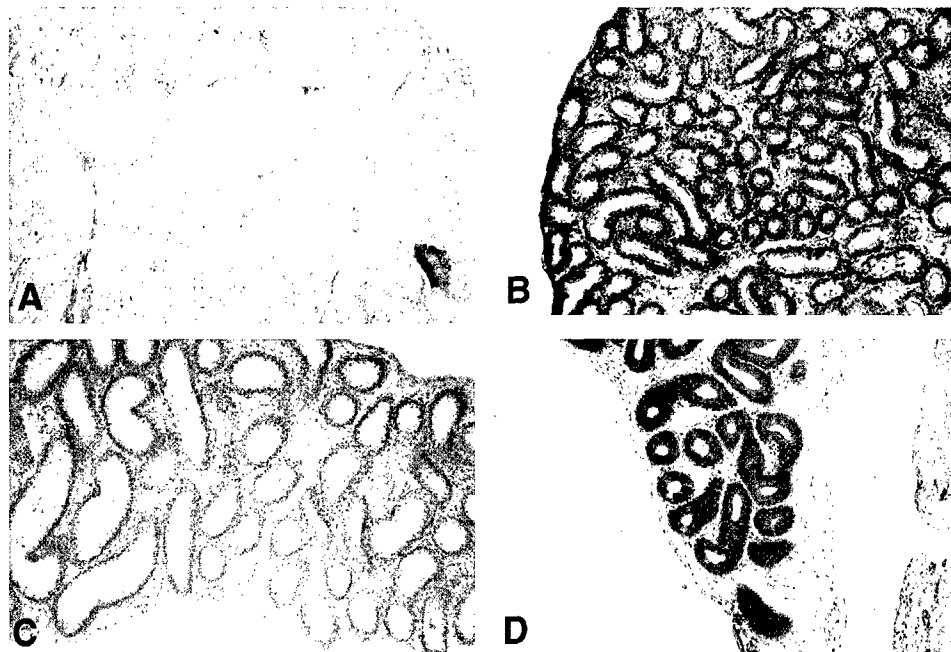


FIG. 1. Photomicrographs of mouse epididymis sections immunostained for negative (treated with preimmune serum) and positive (treated with primary antibody) controls for TGF- α (A and B) and TGF- β (C and D). Reduced from $\times 10$.

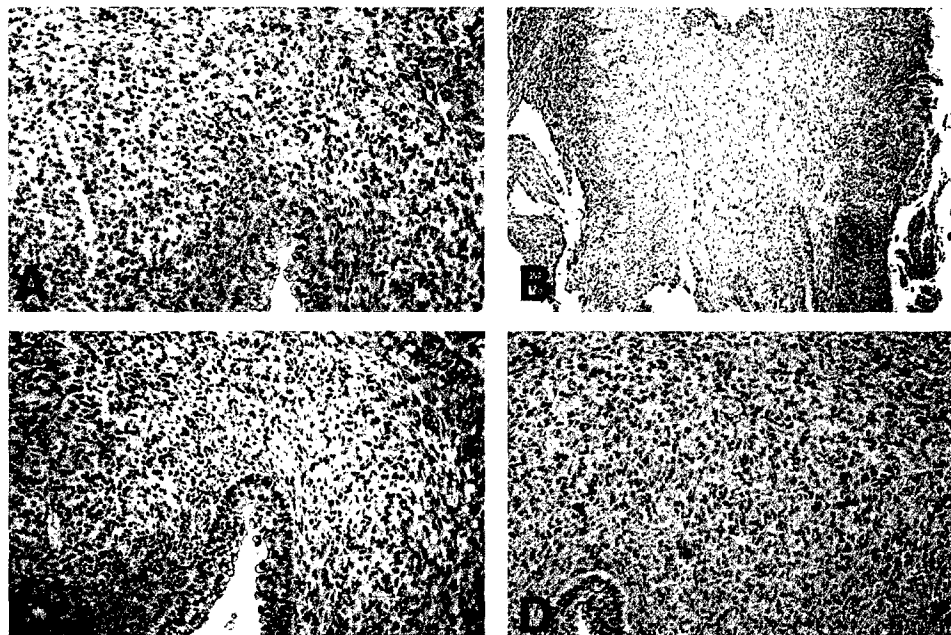


FIG. 2. Photomicrographs of immunostained histological sections of developing human fetal prostate at 9.5 weeks of gestation for TGF- α (A), TGF- $\beta 1$ (B), TGF- $\beta 2$ (C) and TGF- $\beta 3$ (D). 9.5 weeks, $20\times$. A, C and D, reduced from $\times 20$. B, reduced from $\times 10$.

stromal and epithelial interactions.¹³ 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- $\beta 1$, TGF- $\beta 2$ and TGF- $\beta 3$ 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,¹ serum testosterone concentrations peak at about 13 to 16 weeks and gradually decline to female testosterone levels.¹⁴ 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.^{7,15}

Expression of the enzyme 5 α -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.¹⁶ Inhibition of 5 α -reductase enzyme in the male rat results in feminization of the external genitalia and urethra, and partial inhibition of prostatic development. In man the 5 α -reductase deficiency syndrome is recognized as male pseudohermaphroditism characterized by a small or undetectable prostate.¹⁷ Consequently, 5 α -reductase and DHT are critical for normal human prostate development. In humans androgen receptor

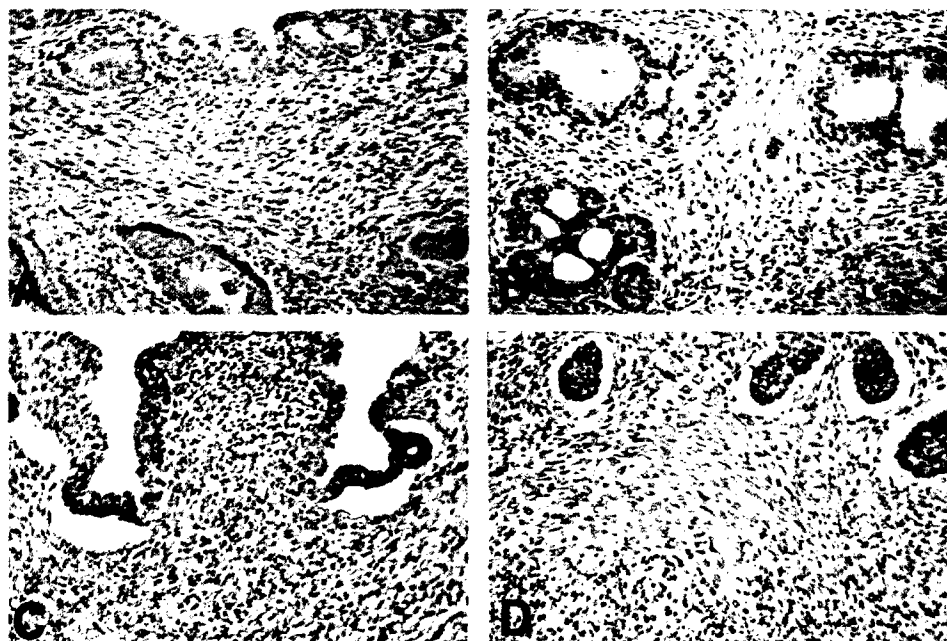


FIG. 3. Photomicrographs of immunostained histological sections of developing human fetal prostate for TGF- α at 16.5 weeks of gestation (A), TGF- β 1 at 16.5 weeks (B), TGF- β 2 at 13 weeks (C) and TGF- β 3 at 13 weeks (D). A, C and D, reduced from $\times 20$. B, reduced from $\times 10$.

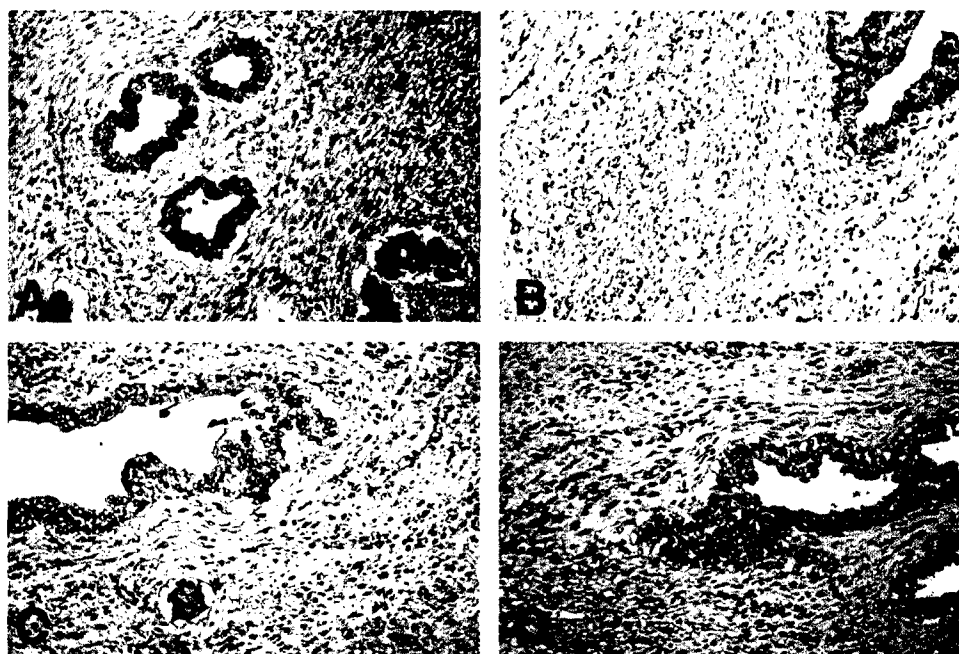


FIG. 4. Photomicrographs of immunostained histological sections of developing human fetal prostate at 20 weeks of gestation for TGF- α (A), TGF- β 1 (B), TGF- β 2 (C) and TGF- β 3 (D). A, C and D, reduced from $\times 20$. B, reduced from $\times 10$.

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.^{18,19} In these experiments if the corresponding urogenital mesenchyme lacks androgen receptor as in the testicular feminization syndrome the prostate does not develop, whereas androgen receptor positive urogenital sinus mesenchyme was able to induce androgen receptor negative testicular feminization epithelium to develop into epithelium. Thus, the presence of

androgen receptor and DHT is necessary to stimulate mesenchymal elaboration of stromal factors. Our studies show that some of those factors are members of the EGF and TGF- β families. TGF- α , TGF- β 1 and TGF- β 3 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- β 2 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- β 1 and TGF- β 3. 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- β 3 in the epithelium during 13 to 16.5 weeks of gestation. Although TGF- α , TGF- β 1 and TGF- β 3 were present initially in the mesenchyme, they later appeared in the epithelium during the androgen surge. Hence, it appears that initially DHT only indirectly influences prostatic epithelium by direct induction of mesenchymal factors that diffuse and affect the epithelium in a paracrine fashion.

The level of TGF- β 1 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- β 1 level seems to suggest down regulation of TGF- β 1 by androgens during the period of active fetal prostate development. TGF- β is primarily a growth inhibitor and antagonizes other stimulatory growth factors but not much is known about the differential roles of the specific TGF- β isoforms. Our studies showed higher levels of TGF- α and TGF- β 3 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.²⁰ Overexpression of TGF- α in transgenic mice results in hyperplasia of the anterior prostate.²¹ Whereas TGF- α has been shown to be a growth stimulator that may be critical in the cellular proliferation associated with prostatic growth, TGF- β 3 may be an important factor in continued ductal elongation and morphogenesis. However, the role of TGF- β 2 in the developing epithelium remains unclear. Recent studies on rat ventral prostate development reveal that TGF- β 1, TGF- β 2 and TGF- β 3 are differentially regulated, that is TGF- β 1 and TGF- β 2 mRNA expression was enhanced while TGF- β 3 mRNA was significantly suppressed after castration. Moreover, the expression of TGF- β 2 and TGF- β 3 was inversely related.²² In other experiments TGF- β 2 null mice have been shown to have multiple developmental defects including urogenital anomalies with no phenotype overlap with TGF- β 1 or TGF- β 3 null mice, suggesting distinct regulatory mechanisms and roles for these isoforms.²³

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

1. Jirasek, J. E.: The relationship between the structure of the testis and differentiation of the external genitalia and phenotype in man. In: *Endocrinology of the Testes*. Ciba Symposium, **16**: 3, 1967.
2. Xia, T. G., Blackburn, W. R. and Gardner, W. A., Jr.: Fetal prostate growth and development. *Ped. Path.*, **10**: 527, 1990.
3. Cunha, G. R.: Epithelio-mesenchymal interactions in primordial gland structures which become responsive to androgenic stimulation. *Anat. Rec.*, **172**: 179, 1972.
4. Takeda, H. and Chang, C.: Immunohistochemical and in-situ hybridization analysis of androgen receptor expression during the development of the mouse prostate gland. *J. Endocr.*, **129**: 83, 1991.
5. Tenniswood, M.: Role of epithelial-stromal interactions in the control of gene expression in the prostate: an hypothesis. *Prostate*, **9**: 375, 1986.
6. Chang, S. M. and Chung, L. W. K.: Interaction between prostatic fibroblast and epithelial cells in culture: role of androgen. *Endocrinology*, **125**: 2719, 1989.
7. McKeehan, W. L., Adams, P. S. and Rosser, M. P.: Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. *Cancer Res.*, **44**: 1998, 1984.
8. Taylor, T. B. and Ramsdell, J. S.: Transforming growth factor- α and its receptor are expressed in the epithelium of the rat prostate gland. *Endocrinology*, **133**: 1306, 1993.
9. Steiner, M. S.: Review of peptide growth factors in benign prostatic hyperplasia and urologic malignancy. *J. Urol.*, **153**: 1085, 1995.
10. Tutrone, R. F., Jr., Ball, R. A., Ornitz, D. M., Leder, P. and Richie, J. P.: Benign prostatic hyperplasia in a transgenic mouse: a new hormonally sensitive investigatory model. *J. Urol.*, **149**: 633, 1993.
11. Kyprianou, N. and Isaacs, J. T.: Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. *Mol. Endocr.*, **3**: 1515, 1989.
12. Donjacour, A. A. and Cunha, G. R.: The effect of androgen deprivation on branching morphogenesis in the mouse prostate. *Dev. Biol.*, **128**: 1, 1988.
13. Story, M. T.: Polypeptide modulators of prostatic growth and development. *Cancer Surv.*, **11**: 123, 1991.
14. Reyes, F. I., Boroditsky, R. S., Winter, J. S. and Faiman, C.: Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations. *J. Clin. Endocr. Metab.*, **38**: 612, 1974.
15. Alarid, E. T., Cunha, G. R., Young, P. and Nicoll, C. S.: Evidence for an organ- and sex-specific role of basic fibroblast growth factor in the development of the fetal mammalian reproductive tract. *Endocrinology*, **129**: 2148, 1991.
16. George, F. W. and Peterson, K. G.: 5α -dihydrotestosterone formation is necessary for embryogenesis of the rat prostate. *Endocrinology*, **122**: 1159, 1988.
17. Imperato-McGinley, J., Binienda, Z., Arthur, A., Mininberg, D. T., Vaughan, E. D., Jr. and Quimby, F. W.: The development of a male pseudo-hermaphroditic rat using an inhibitor of the enzyme 5α -reductase. *Endocrinology*, **116**: 807, 1985.
18. Cunha, G. R., Donjacour, A. A., Cooke, P. S., Mee, S., Bigsby, R. M., Higgins, S. J. and Sugimura, Y.: The endocrinology and developmental biology of the prostate. *Endocr. Rev.*, **8**: 338, 1987.
19. Cunha, G. R. and Donjacour, A. A.: Mesenchymal-epithelial interactions in the growth and development of the prostate. *Cancer Treat. Res.*, **46**: 159, 1989.
20. Derynck, R., Lindquist, P. B., Bringman, T. S., Wilcox, J. N., Elder, J. T., Fisher, G. L., Vorhees, J. J., Moses, H. L., Pittelkow, M. R. and Coffey, R. J., Jr.: Expression of the transforming growth factor-alpha gene in tumor cells and normal tissue. *Cancer Cells*, **7**: 297, 1989.
21. Sandgren, E. P., Luetke, N. C., Palmiter, R. D., Brinster, R. L. and Lee, D. C.: Overexpression of TGF α in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast. *Cell*, **61**: 1121, 1990.
22. Itoh, N., Patel, U., Cupp, A. S. and Skinner, M. K.: Developmental and hormonal regulation of transforming growth factor-beta1 (TGF β 1), -2, and -3 gene expression in isolated prostatic epithelial and stromal cells: epidermal growth factor and TGF β interactions. *Endocrinology*, **139**: 1378, 1998.
23. Sanford, L. P., Ormsby, I., Gittenberger-de-Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L. and Doetschman, T.: TGF β 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF β knockout phenotypes. *Development*, **124**: 2659, 1997.