Age-dependent histopathological findings in the prostate of probasin/SV40 T antigen transgenic rats: Lack of influence of carcinogen or testosterone treatment

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Sequential changes in the phenotype of prostatic lesions and the impact of additional carcinogen treatment or castration on development and progression of prostate cancers were examined in probasin/simian virus 40 (SV40) T antigen transgenic (TG) rats. Non-invasive prostate adenocarcinomas were evident in all lobes at 15 weeks of age. Invasive tumors were limited to the anterior lobe at this time point and were found in all lobes in an age-dependent manner thereafter. No metastasis was apparent at any age. Additional carcinogen treatment or castration did not enhance progression or generate selective growth of hormone-independent prostate cancer cells. These results suggest that our TG rats are suitable for clarification of mechanisms in early stages of prostate carcinogenesis, that is, from prostatic intraepithelial neoplasia (PIN) to non-invasive and then invasive lesions. (Cancer Sci 2003; 94: 153–157)

Prostate cancer is the most common cancer and the second leading cause of death from cancer among US men.[1] It has been estimated there will be approximately 189 000 new cases of prostate cancer and 30 200 deaths from prostate cancer in the United States in 2002.[2] In Japan, the prevalence of prostate cancer has also been increasing, along with changes in life style and lifespan.[3, 4] Although the majority of prostate cancers initially respond to androgen ablation therapy because of hormone-dependent growth,[5] relapse with the generation of hormone-independent cancer cells occurs within 1–2 years and eventually leads to a fatal outcome in many cases.[6, 7] Despite this serious situation, little is yet known about the etiology, risk factors and molecular mechanisms of progression of prostate cancer.

Responding to the need for *in vivo* model systems that adequately reproduce the spectrum of human prostate cancers, we have established an animal model whereby 3,2′-dimethyl-4-aminobiphenyl (DMAB) administration induces ventral prostate carcinomas which are microscopic in size, non-invasive and androgen-dependent,[8–10] while additional long-term treatment with testosterone propionate (TP) causes development of invasive and metastasizing androgen-independent adenocarcinomas, arising from the dorsolateral and anterior prostate and seminal vesicles.[10] However, a long period of about 60 weeks is required to induce prostate cancers and the frequency of lesion development is relatively low. Therefore, we have established transgenic (TG) rats bearing a probasin promoter/simian virus 40 (SV40) T antigen construct in which androgen-dependent prostate cancers develop rapidly.[11] In the present study, we examined sequential changes of prostate lesions histopathologically and assessed whether additional carcinogen or testosterone exposure might result in an androgen-independent phenotype or metastatic lesions.

Materials and Methods

Chemicals and animals. TP was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) hydrochloride and DMAB were obtained from the Nard Institute (Osaka). N-Methyl-N-nitrosourea (MNU) was from Sigma Chemical Co. (St. Louis, MO). Male heterozygous TG rats established in our laboratory with a Sprague-Dawley genetic background were used in the present study.[11] They were housed 3/cage on wood-chip bedding in an air-conditioned animal room at 23±2°C and 50±10% humidity. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*.

Experiment 1. A total of 35 heterozygous male TG rats were sequentially killed at 15, 20, 25, 30, 35, 40 and 45 weeks of age. Six TG rats underwent exogenous hormonal administration from 14 weeks of age for 31 weeks and were killed at 40 and 45 weeks of age. TP was introduced into 2-cm-long Silastic tubes (Dow Corning Co., Midland, MI, inner diameter, 2 mm; outer diameter, 3 mm; approximately 40 mg), which were sealed at both ends with Silastic medical grade adhesive (Dow Corning Co.) and implanted into the subcutis of the interscapular region under anesthesia with ethyl ether. The TP-filled implants were replaced at 6-week intervals.

Experiment 2. A total of 40 heterozygous male TG rats at 5 weeks of age were randomly divided into eight groups. Animals in groups 1 and 5 received intragastric intubations of PhIP at 200 mg/kg body weight once a week from weeks 5 to 7, and those in groups 2 and 6 were treated with subcutaneous injections of DMAB at 50 mg/kg body weight in the same manner. Rats in groups 3 and 7 were given two intraperitoneal administrations of MNU at 50 mg/kg body weight from weeks 5 to 6. In groups 4 and 8, rats were maintained until 35 weeks old. In groups 5, 6, 7 and 8, animals also underwent TP administration from the start of the experiment for 30 weeks, as in experiment 1. All surviving rats were killed at experimental week 30 and subjected to complete autopsy. The proportions of areas with different differentiation grades in each tumor were quantitatively measured with an Image Processor for Analytical Pathology (IPAP) (Sumika Technos Co., Osaka).

Experiment 3. A total of 50 heterozygous male TG rats of 5 weeks of age were randomly divided into five groups. Rats in groups 1 and 3 were subjected to bilateral orchiectomy under ether anesthesia at weeks 30 and 10, respectively. Animals in groups 2 and 4 were continuously treated with ethinyl estradiol (EE), introduced into 0.5-cm-long Silastic tubes (approximately 10 mg EE), from weeks 30 and 10, respectively, to the end of

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the experiment. The hormone-filled implants were replaced at 6-week intervals. All surviving animals, including rats in group 5 as a control, were killed at week 45. Testosterone levels in serum were analyzed by radioimmunoassay at a commercial laboratory (SRL, Tokyo).

Immunohistochemical analysis. The avidin-biotin-peroxidase complex (ABC) method was used to determine the expression of SV40 T antigen, androgen receptor (AR), and Ki-67 in prostate epithelial cells. Prostate sections were treated with mouse anti-SV40 T antigen (1:250, Pharmingen, San Diego, CA), rabbit anti-AR (1:200, Affinity Bioreagents, Golden, CO), and rabbit anti-Ki-67 (1:300, Novocastra Laboratories, Ltd., Newcastle, UK) and then sequentially with secondary antibody and ABC (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). The sites of peroxidase binding were demonstrated with diaminobenzidine as the substrate. For analysis of cell proliferation, Ki-67 labeling indices were generated by counting over 1000 prostate epithelial cells in each lobe under a microscope at high magnification, and were expressed as numbers of Ki-67-positive cells per 100 prostate epithelial cells.

Statistical analysis. Statistical analysis of differences between means and incidences was carried out using analysis of variance (ANOVA) and the Kruskal-Wallis test, respectively. When positive results were obtained, the Mann-Whitney *U* test with Bonferroni correction was applied to evaluate the statistical significance between treatment groups.

Table 1. Sequential histopathological findings of neoplastic lesions in TG rats

Age (wks)	TP	No. of rats	No. of rats with prostatic lesions								No. of rats with tongue tumor[2)]
			Ventral		Dorsal		Lateral		Anterior		
			PIN	Ca	PIN	Ca	PIN	Ca	PIN	Ca	
15	–	5	5	4 (0)	5	2 (0)	5	4 (0)	5	5 (5)	2
20	–	4	3	2 (0)	4	0	4	1 (0)	4	4 (4)	4
25	–	5	5	5 (0)	5	3 (0)	5	5 (0)	5	5 (5)	1
30	–	4	4	4 (0)	4	4 (2)	4	4 (2)	4	4 (4)	2
35	–	5	4	5 (1)[1)]	4	5 (5)[1)]	4	5 (5)[1)]	4	5 (5)[1)]	3
40	–	3	2	3 (2)[1)]	2	3 (3)[1)]	2	3 (3)[1)]	2	3 (3)[1)]	2
40	+	2	2	2 (0)	2	2 (2)	2	2 (2)	2	2 (2)	1
45	–	3	3	3 (3)	3	3 (3)	3	3 (3)	3	3 (3)	2
45	+	4	4	4 (4)	4	4 (4)	4	4 (4)	4	4 (4)	3

PIN, prostatic intraepithelial neoplasia; Ca, carcinoma; number of rats with invasive carcinoma in parentheses.
1) One case was diagnosed as a small cell carcinoma.
2) All tumors were small cell carcinomas.

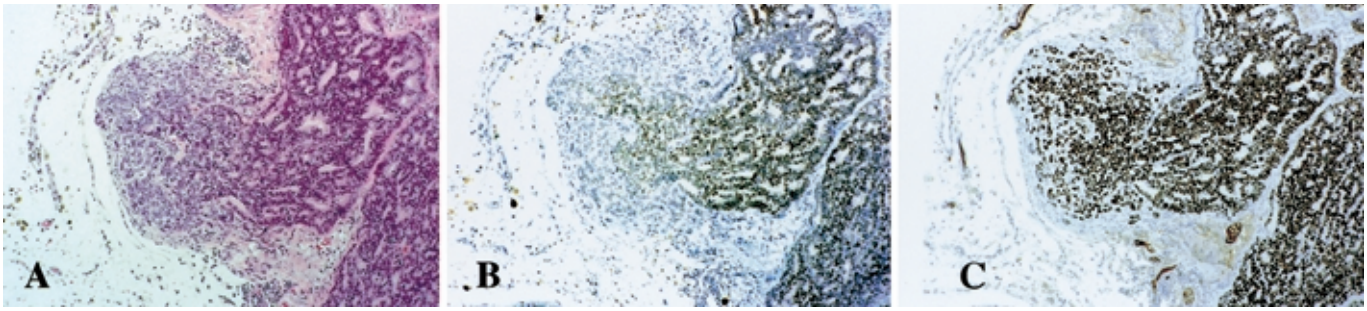

Fig. 1. Representative findings for AR and SV40 T antigen protein expression in serial sections of a microinvasive adenocarcinoma of the ventral prostate in a 35-week-old TG rat. A: H&E. B: AR. C: SV40 T antigen.

Table 2. Histopathological findings of neoplastic lesions in TG rats given additional carcinogen treatement

Group	Treatment		No. of rats	Prostate gland						AC in mammary gland		LN metastasis of S		S in tongue
	TP	Carcinogen		Ventral		Dorso-lateral		Anterior		Incidence	Multiplicity (No./rat)	Incidence	Multiplicity (No./rat)	
				AC	S	AC	S	AC	S					
1	–	PhIP	3	3	0	1	1	2	0	0	0	1	0.67±1.15	1
2	–	DMAB	4	4	0	4	0	4	0	1	0.25±0.50	2	1.00±1.41	1
3	–	MNU	5	5	0	5	0	5	0	2	0.40±0.55	1	0.20±0.45	1
4	–	Control	4	4	0	4	0	4	0	0	0	0	0	0
5	+	PhIP	4	4	0	4	1	4	0	4	5.00±1.15	1	1.00±2.00	2
6	+	DMAB	3	3	0	3	0	3	0	1	1.00±1.73	0	0	0
7	+	MNU	2	2	0	2	0	2	0	2	5.00±1.41	0	0	0
8	+	Control	4	4	0	4	0	4	0	2	0.75±0.96	0	0	1

TP, testosterone propionate; AC, adenocarcinoma; S, small cell carcinoma; LN, lymph node.

Results

Experiment 1. To examine the development of preneoplastic and neoplastic lesions in prostate glands, TG rats were sequentially killed every 5 weeks starting at 15 weeks of age. The observed histopathological lesions of the prostate were classified as described previously[11] into prostatic intraepithelial neoplasia (PIN) and adenocarcinoma categories. The histopathological findings for prostatic lesions are summarized in Table 1. PINs were detected in all lobes of all animals examined at 15 weeks of age. Non-invasive adenocarcinomas were already present in all lobes with high incidences, while invasive carcinomas were observed only in the anterior prostate at the first time point. However, invasive cancers in other lobes also increased in an age-dependent manner thereafter. Some invasive cancers demonstrated a lack or decrease of AR, while SV40 T antigen protein was expressed at a high level (Fig. 1). There were no metastatic lesions from prostate adenocarcinomas at any age. Single 35- and 40-week-old rats had large small cell carcinomas invading beyond the prostate lobes. Malignant tongue tumors characterized by small round cells and demonstrating metastasis to regional lymph nodes were also observed in some TG rats (details published in Ref. 12).

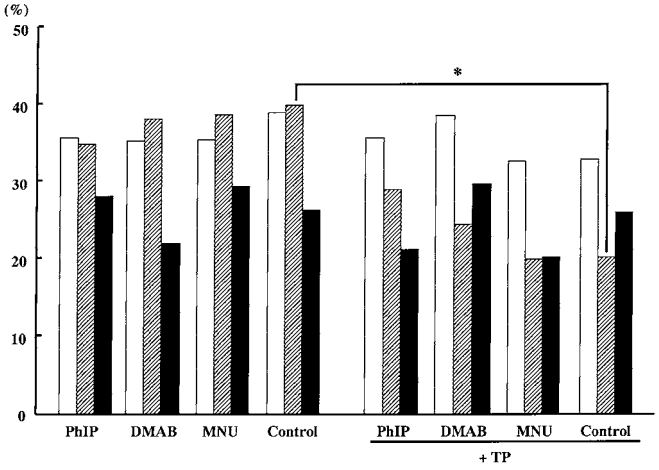

Fig. 2. Ki-67 labeling indices for prostate adenocarcinoma cells in TG rats given additional prostatic carcinogen treatment. ☐ ventral, ▨ dorsolateral, ■ anterior lobes. * Significantly different at $P<0.05$.

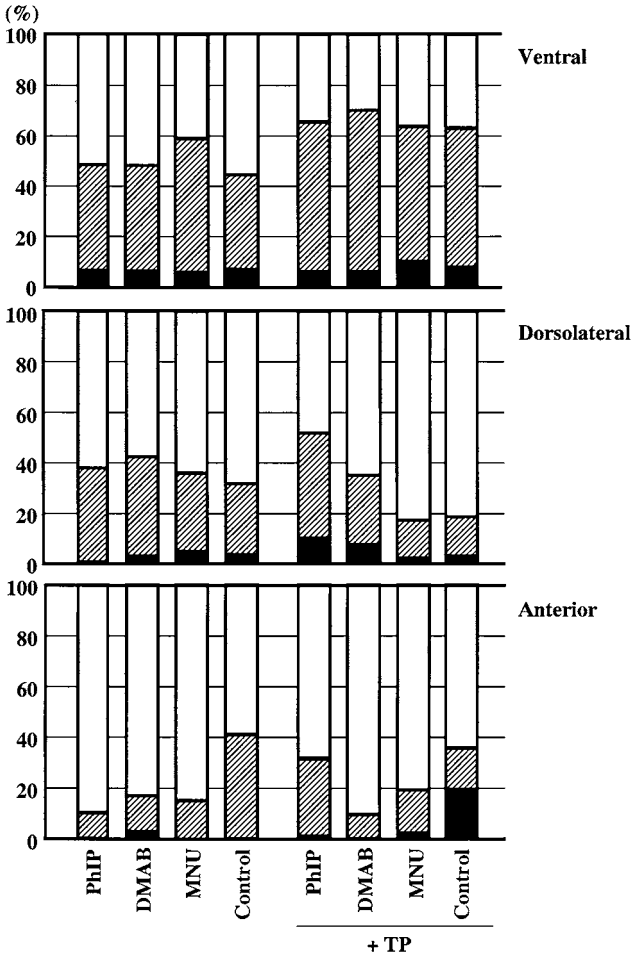

Fig. 3. Results of quantitative analysis of the proportions of different differentiated components in prostate cancer lesions in TG rats given additional prostatic carcinogen treatment. ☐ well, ▨ moderately, ■ poorly differentiated adenocarcinoma components.

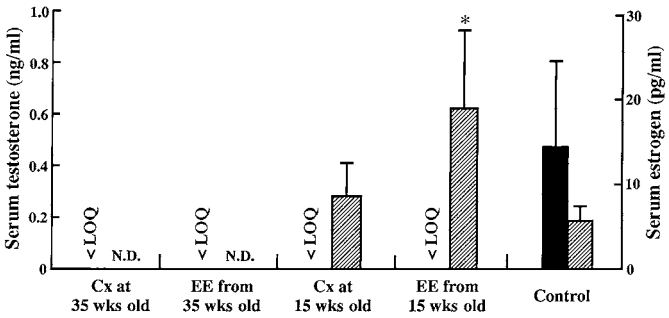

Fig. 4. Effects of bilateral orchiectomy or EE treatment on serum testosterone (■) and estradiol (▨) levels in TG rats. * Significantly different from the control value at $P<0.05$. EE, ethinyl estradiol; Cx, bilateral orchiectomy; ND, not determined; LOQ, limit of quantitation.

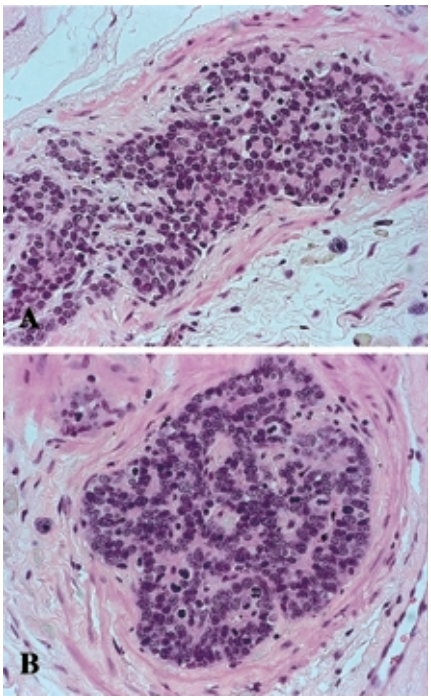

Fig. 5. Representative findings for involuted prostate cancer lesions in TG rats treated with bilateral orchiectomy (A, group 1) and EE (B, group 2) at 35 weeks of age. Original magnification, ×400.

TP treatment had no effect on any of the progressive changes of prostate cancers, that is, the frequency of preneoplastic and neoplastic lesion development in prostate glands was almost the same as those of non-TP-treated rats, and no metastatic adenocarcinomas were found.

Experiment 2. To investigate the effects of additional administration of genotoxic carcinogens on prostate cancer progression, TG rats were treated with PhIP, DMAB or MNU. The histopathological findings are summarized in Table 2. Metastatic lesions to lymph nodes of only small cell carcinomas, not adenocarcinomas, were found in a few cases. Representative results of Ki-67 immunohistochemical staining of prostate cancer cells are shown in Fig. 2. There were no obvious intergroup differences in labeling indices in cancerous lesions of any lobes in either non-TP- or TP-treated rats. All TG rats in this study contained two or three different differentiation components in adenocarcinomas and the proportions of each were quantitatively analyzed in single lesions. No differences could be found in the percentages of each histological component among the groups (Fig. 3). Interestingly, mammary adenocarcinomas were observed in some of the TG rats and TP promoted their development (Table 2).

Experiment 3. From the results of experiment 1, microinvasive adenocarcinomas might be androgen-independent because of the observed decrease or lack of AR protein expression. The possibility of persistence of cancer cells after androgen ablation treatment was therefore investigated. Serum testosterone data are shown in Fig. 4. All castrated rats undergoing either bilateral orchiectomy or subcutaneous implantation of EE-containing tubes demonstrated testosterone levels below the limit of quantitation. Involuted cancer cells were found with an associated abundant stromal tissue in all lobes of prostate glands in both rats given bilateral orchiectomy and those given EE treatment (Fig. 5). There were no remarkable differences in involuted cancer cell morphology between the different treatment groups. No recurrent cancer lesions were observed in any treated group.

Discussion

The present study confirmed sequential development of prostate lesions in our TG rats, but a lack of any effects of additional genotoxic carcinogen or testosterone influence on adenocarcinoma progression. Earlier we had found high-grade PINs from 4 weeks of age,[11] while those lesions only occurred between 10–12 weeks of age in the transgenic adenocarcinoma mouse prostate (TRAMP) model.[13] By 24–30 weeks, all TRAMP males spontaneously develop primary prostate tumors with metastasis commonly detected in regional lymph nodes and lung.[14] In our TG rat model, while invasive adenocarcinomas develop in all lobes by 30–35 weeks of age, metastasis is not observed until the animals are 45 weeks old, although metastatic lesions from malignant tongue tumors are found.[12] The SV40 T antigen used in TG acts as a potent oncoprotein through inhibiting both the pRB and p53 tumor suppressor pathways.[15] However, this may have no relation to metastasis of prostate adenocarcinoma. The metastasis suppressors, KAI1 and CD44, are known to contribute to metastasis of human prostate cancer when down-regulated,[16, 17] but to our knowledge there have been no reports of their transcriptional regulation by pRB or p53. A p53-binding site has been identified in the promoter region of the *KAI1* gene, but it was demonstrated that the presence of a p53-binding site in regulatory domains is in itself not sufficient for definition as a *p53* transcriptional target gene.[18, 19] The lack of metastases from prostate adenocarcinomas in our TG rat may be due to the existence of pathways that can inhibit metastasis. Small cell carcinomas were also occasionally observed in the prostates, lymph nodes and tongue of TG rats, reminiscent of the poorly differentiated metastatic carcinomas noted in the TRAMP mouse using the same gene construct.[20] However, such small cell carcinomas in the TG rats demonstrated neuroendocrine tumor characteristics, such as synaptophysin[21] and protein gene product 9.5 expression,[22] which were lacking in the TRAMP mice. Therefore, the origins of well-differentiated adenocarcinomas and small cell carcinomas may be very different, and development of small cell carcinomas is probably not a result of progression from well-differentiated adenocarcinomas.[11]

In the present study, SV40 T antigen, which is regulated by the androgen-dependent probasin promoter, was expressed in all microinvasive prostate carcinomas, while some of these lesions had reduced or absent AR expression. There is a big difference in the time-course for disappearance of protein expression in prostate cancer between AR and SV40 T antigen after castration, that is, 1 day for AR and 7 days for SV40 T antigen.[11] The difference in half-lives between these proteins may be the reason for this phenomenon.

It is widely accepted that the accumulation of multiple genetic and epigenetic alterations is intimately involved in prostate tumor development.[23] However, in the present study, additional treatment with prostatic carcinogens, PhIP, DMAB or MNU, known to induce genetic alterations,[24–26] did not exert any influence on prostate cancer progression assessed in terms of histological grade or metastatic ability. These data suggest that secondary epigenetic changes play an important role in prostate cancer progression rather than genetic events. In this context, it is of interest that recent studies have shown that a frequent down-regulation of CD44 and KAI1 expression is associated with allelic loss rather than point mutation.[27, 28] We previously demonstrated that down-regulation of AR protein expression might be involved in the progression stage[29] and aberrant hypermethylation in the AR promoter region may play a critical role in AR expression in rat prostate carcinomas.[30] The fact that lack of AR protein expression was here observed in some microinvasive adenocarcinomas provides support for this speculation.

Endogenous androgen blockade therapy is widely accepted as a treatment for human prostate cancers.[31, 32] However, the outgrowth of hormone-independent cancer cells is a frequent outcome and the existence of an AR-negative subpopulation predicts a poor response to androgen blockade therapy.[33] AR-negative cancer cells were often found in microinvasive prostate cancer foci in our TG rats of 35 weeks of age, but our investigation of the possibility of androgen-independent cancer development after castration here resulted in severe involution of all adenomatous lesions. Several factors other than androgens are reported to activate AR transcriptional activity, such as IGF-I, EGF, KGF, IL6 and forskolin, and these are considered to play a critical role in the selective growth of hormone-independent cancer cells.[34] Their manipulation might improve the potential of our TG rats as an animal model for elucidation of the molecular mechanisms of prostate cancer progression. Further studies in this direction thus appear warranted.

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