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Trial to Induce Prostatic Cancer in ACI/Seg Rats Treated with a Combination of 3,2′-Dimethyl-4-aminobiphenyl and Ethinyl Estradiol

Kazuhiro Takai,[1,3] Tadao Kakizoe,[2] Yoshinori Tanaka,[2] Ken-ichi Tobisu[2] and Yoshio Aso[1]

[1]*Department of Urology, Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113 and* [2]*Urology Division, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104*

In an attempt to induce prostatic adenocarcinoma at higher incidence in a shorter period, we administered diet containing 0.75 ppm of ethinyl estradiol (EE) for three weeks to ACI/Seg rats, which are predisposed to develop a high incidence of microscopic adenocarcinoma of the prostate at higher age. Then, feeding was changed to basal diet and a single subcutaneous injection of 50 mg/kg body weight of 3,2′-dimethyl-4-aminobiphenyl (DMAB) was given two days after the change. We repeated this schedule 10 times. The rats were killed in week 60 of the experiment and subjected to routine autopsy. The average body weight of rats in group 1 given EE and DMAB was lower than that of control rats in group 2. The incidence of adenocarcinoma was not significantly different in the two groups, i.e., 6/74 (8.1%) in group 1 and 2/54 (3.7%) in group 2. The lesions were all microscopic. The incidence of atypical hyperplasia was significantly higher in group 1 at 17 of 74 rats (23.0%) whereas in group 2, it was only 2 of 54 rats (3.7%). Simple hyperplasia was also observed in 25 of 74 rats (33.8%) in group 1, which was significantly higher than that in group 2, where six of 54 rats (11.1%) had this lesion. The reduced growth of animals due to treatments with EE and DMAB probably suppressed the development of prostate cancer in this experiment. Further studies are needed to develop an appropriate model to induce prostate carcinoma at higher incidence in a shorter period.

Key words: Prostatic cancer — ACI/Seg rats — 3,2′-Dimethyl-4-aminobiphenyl — Ethinyl estradiol

The carcinogenic process of the prostate has not been studied extensively. Sequential analyses of the carcinogenesis in animals would be helpful in understanding the processes of differentiation and progression of prostate cancer in humans. However, no good animal model of prostate cancer has yet been established. Bosland *et al.*[1] reported the induction of visible prostatic adenocarcinomas in 5 of 20 Wistar rats 79 weeks following a single injection of 50 mg/kg NMU.[4] The rats had been pretreated with a daily dose of 50 mg/kg cyproterone acetate for three weeks and then three daily injections of 100 mg/kg testosterone. We tried to induce prostatic adenocarcinoma in Fischer 344 rats by our modified method,[2] but observed well-differentiated adenocarcinoma in only 3 of 54 animals (5.6%), atypical hyperplasia in 19% and simple hyperplasia in 88% in week 60.

Shirai *et al.*[3] reported that when male Fischer 344 rats were given a diet containing 0.75 ppm of EE for 3 weeks and then basal diet for 2 weeks alternately 10 times, and a single subcutaneous injection of 50 mg/kg body weight of DMAB 2 days after each change to basal diet, prostatic carcinomas developed in 18 of 21 rats (85.7%); the lesions were all microscopic and developed in the ventral lobe of the prostate. However, in a subsequent study they observed a lower incidence of prostatic cancer.[4]

The ACI/Seg rat is known to be a useful animal model for studies on the etiology and pathogenesis of naturally occurring prostatic cancer.[5–8] The inbred ACI strain of rat was initially established by Curtis and Bullock in 1926 by cross breeding an inbred August (AUG; "A") with an inbred Copenhagen (COP; "C") rat.[8] Since the inbred ACI/Seg rat was originally derived from a Copenhagen rat, these 2 inbred strains were initially genetically very similar.[8] However, male ACI/Seg rats, unlike Copenhagen rats, show a high incidence of both microscopic and gross cancer of the ventral prostate. The ACI/Seg strain was established as a permanent, defined, flora isolator colony and was developed by a commercial laboratory animal producer (Harlan Industries, Cumberland, Ind.). At 24 months of age, 35 to 45% of ACI/Seg rats have intra-alveolar atypical hyperplasias, the earliest lesions. These lesions progress to intra-alveolar cribriform carcinomas which spread along alveoli and ducts. As the tumors enlarge, they become nodular and invade the capsule or adjacent tissues. By 33 months, 95 to 100% of ACI/Seg rats have intra-alveolar prostatic atypical hyperplasias, and 35 to 40% have invasive carcinomas.

Therefore, in an attempt to induce prostatic adenocarcinoma at higher incidence in a shorter period, we administered EE and DMAB to ACI/Seg rats, assuming that they would be more sensitive to chemical carcinogens than ordinary rats.

[3] To whom correspondence should be addressed.

[4] Abbreviations used are: NMU, N-nitroso-N-methylurea; EE, ethinyl estradiol; DMAB, 3,2′-dimethyl-4-aminobiphenyl.

MATERIALS AND METHODS

Animals Male ACI/Seg rats of 22 weeks old were purchased from Harlan Industries, Cumberland, Ind. The experiment was started when the rats were 25 weeks old. The rats were divided into 4 groups and kept in metal cages in a room at 23°C and 50% humidity with a 12 h-12 h light-dark cycle. The 4 animals in each cage were marked by cutting the right or left ear, or both or neither. They were fed *ad libitum,* alternately on CE-2 pellet diet (CLEA Co., Tokyo) and CE-2 powder diet (CLEA Co.) with (experimental group) or without (control group) EE, as described below. They were weighed every two weeks.

Chemicals EE was purchased from Sigma chemical Co., St. Louis, Mo. and DMAB from Matsugaki Pharmaceutical Co., Osaka. A solution of 3 mg of EE in 10 ml of corn oil was mixed with 4 kg of CE-2 powder diet to give a final concentration of 0.75 ppm of EE in the diet (EE diet). The EE diet was shaken in a mixer overnight and then stored at 4°C in a cold room. The animals were given EE diet *ad libitum,* and their intake per day was calculated twice a week. DMAB was diluted in corn oil and the solution (equivalent to 50 mg DMAB/kg body weight) was administered subcutaneously to the rats.

Treatment of animals The experimental schedule and number of animals in each group are shown in Fig. 1. Rats in group 1 were treated as described by Shirai *et al.*[3] One cycle of chemical treatment consisted of administration of EE diet for three weeks and change to basal diet for 2 weeks with a single subcutaneous injection of 50 mg/kg body weight of DMAB two days after the change. This cycle was repeated 10 times. As controls, animals in group 2 were given basal diet and received injections of vehicle only without DMAB.

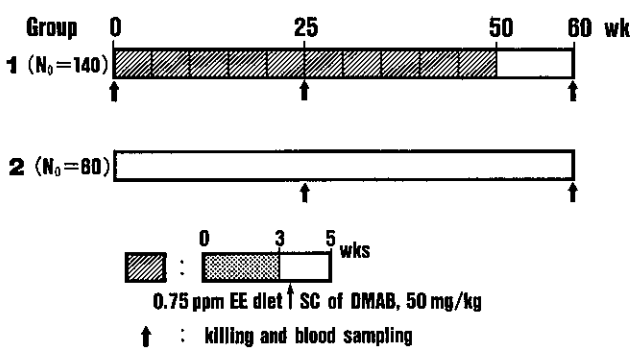

Fig. 1. Experimental protocol. Rats in group 1 were treated as described in Shirai *et al.*'s report.[3] Rats in group 2 were used as controls. The blood testosterone concentration was determined at the beginning of the experiment and in week 25 and week 60.

Blood testosterone concentrations were determined at the beginning of the experiment and in weeks 25 and 60. Samples of about 1 ml of blood were collected from a tail vein with a syringe between 10:00 AM and 12:00 AM and centrifuged, and the serum was stored at −20°C until used for testosterone measurement by radioimmunoassay.[9]

Ten animals were killed at the beginning of the experiment to determine initial values, and 5 in each group were killed in week 25. Surviving rats were killed in week 60 and autopsied. Animals that died or became moribund during the experiment were also autopsied when possible. Portions of the prostate, seminal vesicles, coagulating gland, liver, kidneys, urinary bladder, and grossly abnor-

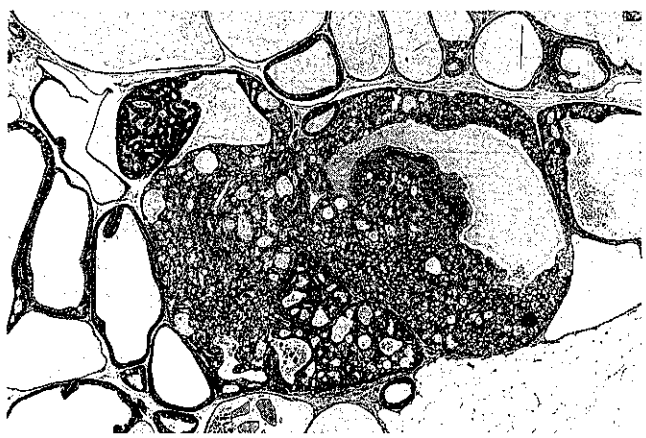

Fig. 2. Prostatic carcinoma composed of atypical cells involving more than one acinus with a cribriform pattern of the glands. H-E, ×13.

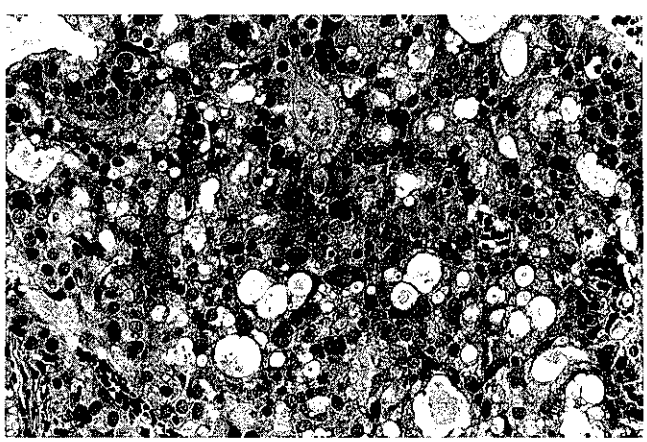

Fig. 3. Portion of Fig. 2 at higher magnification. Note nuclear atypism and mitoses. H-E, ×80.

mal lesions were fixed in 15% buffered formalin, embedded in paraffin and sectioned at 4 μm thickness. Large transverse sections were made to observe the ventral, dorsal and lateral lobes of the prostate and the periurethral areas. The sections were stained with hematoxylin and eosin (H-E) for histological examination.

All specimens were compared histologically with a reference[10] and examined according to the criteria described in our previous report[2] and by Shirai *et al.*[11] Carcinomas were identified as large proliferative lesions composed of atypical cells involving more than one acinus, with a cribriform pattern of glands (Figs. 2 and 3). Atypical hyperplasia was defined as a small proliferative lesion of atypical epithelium (Fig. 4). Simple hyperplasia was defined as slight proliferative growth of the epithelial lining of the acini without any cellular atypia (Fig. 5). Survival rate in rats was calculated by the Kaplan-Meier method and the significance of differences between two groups was evaluated by using the generalized Wilcoxon test. Statistical analysis of differences was done by Bonferroni's multiple comparison method.[12]

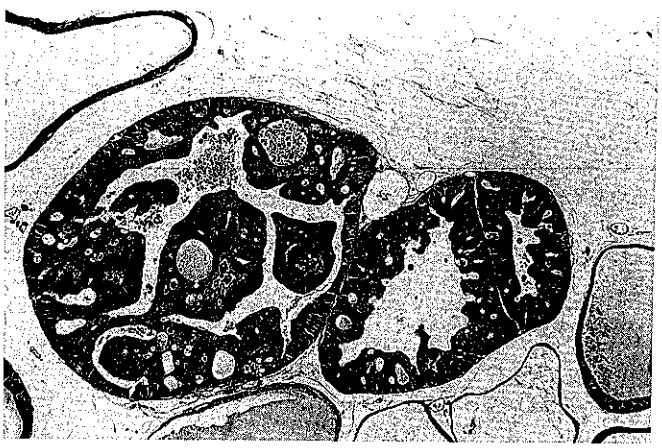

Fig. 4. Atypical hyperplastic lesion of the prostate. Multilayered atypical epithelium is seen. H-E, ×20.

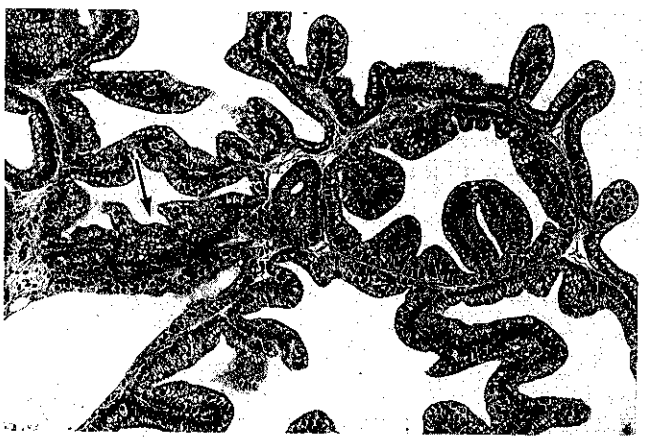

Fig. 5. Proliferative intra-alveolar lesion of the prostate as simple hyperplasia. Some areas progress to atypical hyperplasia (arrow). The epithelium is several cells thick. H-E, ×40.

RESULTS

Food intake and body weight Changes in food intake per day are shown in Fig. 6. The intake in group 1 during administration of EE diet decreased to 3–8 g/day, but returned to 18–20 g/day during administration of CE-2 diet. This decrease in food intake was observed every

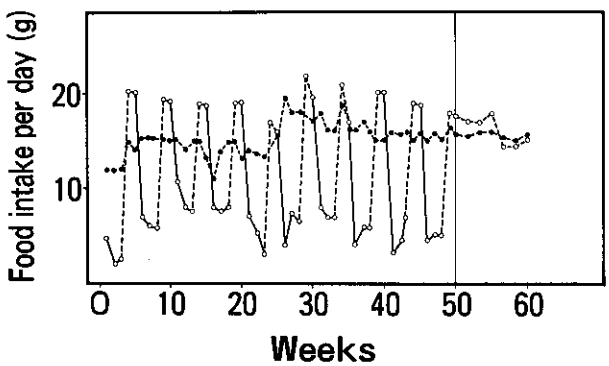

Fig. 6. Changes in food intake per day. The repeated changes in group 1 were due to the toxicities of EE and DMAB. ○, Group 1; ●, Group 2; ——, EE diet; ------, CE-2 diet.

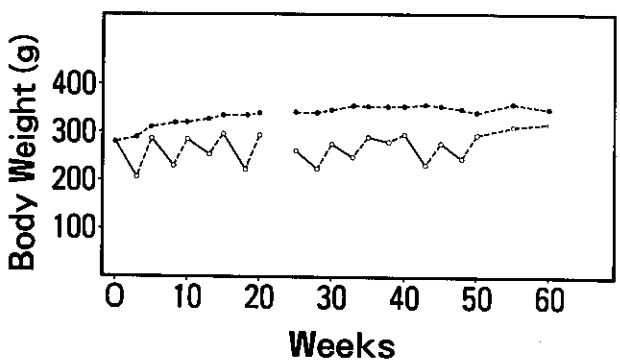

Fig. 7. Changes in body weight. The body weight in group 1 decreased during administration of EE diet, and increased when the diet was changed from EE diet to CE-2 diet. ○, Group 1; ●, Group 2; ——, EE diet; ------, CE-2 diet.

time EE diet was given. The average food intake in group 2 was about 15 g/day and did not change significantly throughout the experiment.

Changes in body weight are shown in Fig. 7. The rats were not weighed between week 20 and week 25. Because of the changes in food intake in group 1, the changes in body weight in the two groups were significantly different. In group 1, the body weight decreased by 20–80 g/cycle during administration of EE diet, but increased when the diet was changed to CE-2 pellets. The average body weight of rats in group 1 given EE and DMAB was lower than that of control rats in group 2 throughout the experiment.

In group 1, the number of effective animals did not decrease appreciably until 30 weeks, but then decreased linearly and rapidly to a final effective number of 74. The cause of death in most animals in which it could be identified, was a tumor of the Zymbal gland in the ear duct: it was not a prostatic tumor in any case. In other cases the cause of death could not be identified because of cannibalism after death. The final number of effective rats in group 2 was 54. There were a significant difference in the 60-month survival rate between groups 1 and 2 as evaluated by means of the generalized Wilcoxon test ($P<0.01$).

Incidences of prostatic lesions, body weights, organ weights and organ-to-body weight ratios Five animals killed at the beginning of the experiment, and 5 of each group in week 25 showed no abnormalities of the prostate.

The incidences of prostatic lesions in week 60 are summarized in Table I. Two types of lesions were seen in some specimens; for example, some showed both adenocarcinoma and atypical hyperplasia, and others both atypical hyperplasia and simple hyperplasia. Adenocarcinoma was induced in 6 of 74 rats (8.1%) in group 1 and two of 54 rats (3.7%) in group 2, the difference in incidences being not significant. The lesions were all microscopic. Some adenocarcinomas were of the cribriform type invading surrounding acini. The adenocarcinomas in group 1 were larger and more multifocal than those in group 2. All lesions listed in Table I were located in the ventral lobe of the prostate gland: no proliferative lesion was found in the dorsolateral lobes. No other proliferative lesions of the accessory sex glands were seen.

Table I. Incidences of Prostatic Lesions in Week 60

Group	Carcinoma	Incidence of prostatic lesion (%)		
		Atypical hyperplasia	Simple hyperplasia	Normal
1 (N=74)	6 (8.1)	17 (23.0)[a]	25 (33.8)[b]	37 (50.0)
2 (N=54)	2 (3.7)	2 (3.7)[a]	6 (11.1)[b]	45 (83.3)

a, b) $P<0.01$ between group 1 and 2.

Table II. Organ Weights and Ratios of Organ Weights to Body Weights in Sub-groups Classified according to Prostatic Lesions

Group	Histology	Body weight (g)	Prostate (g)	Prostate % of body weight	Seminal Vesicle (g)	Seminal vesicle % of body weight
1	Carcinoma	330±28	1.29±0.33	3.90±0.86	1.50±0.38	4.50±0.87
	Atypical hyperplasia	313±54	1.14±0.34	3.60±0.85	1.47±0.56	4.59±1.64
	Simple hyperplasia	322±38	1.18±0.26	3.67±0.74	1.68±0.59	5.22±1.83
	Normal	324±42	1.15±0.26	3.62±0.88	1.60±0.48	4.78±1.41
	Total	322±42[a]	1.17±0.28[a]	3.65±0.82[a]	1.59±0.53[a]	4.86±1.56[a]
2	Carcinoma	375±35	1.47±0.09	3.93±0.14	1.47±0.02	5.19±1.49
	Atypical hyperplasia	345±78	1.20±0.47	3.42±0.59	1.42±0.09	4.18±0.68
	Simple hyperplasia	343±32	1.50±0.50	3.89±0.67	1.57±0.64	4.69±2.22
	Normal	348±34	1.41±0.30	4.06±1.01	1.37±0.43	3.88±1.26
	Total	348±35[a]	1.41±0.32[a]	4.01±0.94[b]	1.40±0.44[b]	4.04±1.40[a]

a) $P<0.01$ between group 1 and 2.
b) $P<0.05$ between group 1 and 2.

Table III. Serum Testosterone Levels in Sub-groups Classified according to Prostatic Lesions

Group	Histology	Testosterone (ng/ml)		
		0 wk	25 wk	60 wk
1	Carcinoma	1.73±1.23	1.88±0.45	3.03±2.16
	Atypical hyperplasia	1.34±0.46	2.46±1.41	2.17±2.65
	Simple hyperplasia	1.67±0.77	3.22±2.47	2.10±1.40
	Normal	1.62±1.25	2.55±1.71	3.02±0.98
	Total	1.62±0.88[a]	2.86±2.20[a]	2.27±1.68[a]
2	Carcinoma	3.6	2.1	1.6
	Atypical hyperplasia	2.30±1.84	1.75±0.50	1.35±0.35
	Simple hyperplasia	2.25±0.95	1.88±0.59	2.82±4.03[b]
	Normal	2.96±2.60	1.92±0.93	1.32±0.63[b]
	Total	2.59±1.95[a]	1.94±0.88[a]	1.50±1.41[a]

a) $P<0.01$ between group 1 and 2.
b) $P<0.05$ between sub-group with simple hyperplasia and normal sub-group 2.

Atypical hyperplasia was induced in 17 of 74 rats (23.0%) in group 1 and 2 of 54 rats (3.7%) in group 2. The difference in incidences was significant ($P<0.01$). Simple hyperplasia was induced in 25 of 74 rats (33.8%) in group 1 and 6 of 54 rats (11.1%) in group 2, the difference in incidences also being significant ($P<0.01$).

The rats in each group were classified into sub-groups according to their prostatic lesions. The organ weights and the organ-to-body weight ratios in these sub-groups are shown in Table II. No significant differences were found in values in the subgroups of either group 1 or 2, or between the values in groups 1 and 2, although the averages of the final body weight and weights of the prostate were significantly lower in group 1 than in group 2 ($P<0.01$), and the average weight of the seminal vesicles was significantly higher in group 1 than in group 2 ($P<0.01$).

Data on the testosterone levels in the blood are shown in Table III. There was no difference between the levels in the subgroups in group 1. However, in group 2 there was a significant difference between the values in the sub-group with simple hyperplasia and the normal sub-group in week 60 ($P<0.05$). There was also a significant difference between the levels in groups 1 and 2 at the three points: the blood testosterone level in group 1 was lower at the beginning of the experiment and higher in week 25 and week 60 than that in group 2.

DISCUSSION

ACI/Seg rats are a good animal model of prostatic adenocarcinoma, because they are known to show high incidences of intraalveolar atypical hyperplasia and microscopic carcinoma, together with occasional invasive or visible carcinoma.[5-8] In the ACI/Seg rat the serum testosterone level remains fairly constant at 3.5–4.0 ng/ml between 4 and 24 months of age, whereas in the male COP rat it decreases continuously from a maximum level of about 3 ng/ml between 3 and 8 months to only about 1 ng/ml at 24 months of age.[8] Between 24 and 36 months of age, male ACI/Seg rats have a 1.5- to 3.5-fold higher serum levels of testosterone than age-matched male COP rats.[8] Thus in the ACI/Seg rats testosterone levels are high while cellular proliferation is vigorous. Noble[13] reported that hormonal factors such as testosterone act as co-carcinogens causing autonomous cell proliferation leading to prostate cancer in experimental animals. The changes described by Noble might occur spontaneously in ACI/Seg rat. However, although ACI/Seg rats show a high incidence of spontaneous prostate cancer, this cancer takes more than 2 years to develop.

Using ACI/Seg rats, we tried to develop an animal model in which a higher incidence of manifest prostatic adenocarcinoma could be induced by a chemical carcinogen in a shorter period. We chose DMAB as the chemical carcinogen because in a previous study[2] we found that NMU was not very effective for inducing carcinomas in the prostate gland. However, although we used susceptible animals, the incidence of adenocarcinoma was only 8.1%, and all the cancers were microscopic. The lower incidence than expected of prostatic adenocarcinoma in this experiment might be explained as follows.

1) In our experiments, EE diet decreased the body weight of animals more severely than reported[3,4] and was thought to be one cause of the high mortality in group 1 during chemical treatment. In the report of Shirai *et al.*[11] their rats that were pretreated with DMAB were especially susceptible to EE toxicity so that the concentration of EE had to be decreased in the middle of the experiment. The depression of growth by treatment with EE and DMAB probably reduced the incidence of prostate cancer in our study. This possibility is supported by reports that during chemical carcinogenesis in other organs, such as the colon and mammary gland, severe restriction of calory intake reduced the incidence of tumors.[14] Soderkivest *et al.*[15] found that in the rat, the ventral prostate contains enzymes that can convert promutagenic compounds to ultimate mutagenic metabolites. DMAB is a carcinogenic aromatic amine and must be activated enzymatically in the prostate to exert its carcinogenicity. Severe suppression of the pros-

tate by EE may reduce the activity of the enzymes for conversion of DMAB to an ultimate mutagen.

Chemical castration was expected to stimulate the prostate by the rebound phenomenon. However, this phenomenon turned out to be not reliable or stable. Chemical castration was thought to induce complete atrophy of the prostate gland. Pollard and Luckert[16] reported that administration of testosterone and NMU without chemical castration induced palpable adenocarcinomas in 6 of 9 (66%) rats within 10.6 months. Our previous study[2] and the present work also indicate that chemical castration is undesirable for the development of cancer.

2) Compared to the reports of Ward *et al.*[7] and Saksena and Lau,[17] we found that the serum testosterone levels in ACI/Seg rats in both groups were lower at the three examining points. We collected serum between 10:00 AM and 12:00 AM, knowing that the testosterone level might show a circadian change. Testosterone might have been partially inactivated during separation of the serum. As the level was also lower even in the control group, the lower level might be explained as being due to the procedure employed to estimate testosterone.

3) The duration of the experiment was too short to allow development of prostate cancer, even in susceptible animals. The reasons why we used ACI/Seg rats at 25 weeks of age in this study were as follows: as our previous report proved the histological change induced by chemical manipulation in rats at 6 weeks of age to disappear after 4 weeks, chemical manipulation in the early phase might not act effectively on prostatic carcinogenesis. Although our observational period was only 60 weeks, ACI/Seg rats at 85 weeks of age could be examined and these older rats were thought to have relatively gross lesions compared with younger rats. As serum testosterone levels in ACI/Seg rats remain high until 24 months, chemical manipulations, which were begun at 25 weeks of age and were repeated until 75 weeks of age, were expected to be effective in inducing prostatic carcinoma under the high level of serum testosterone. Probably, the incidence of prostate cancer would have been higher and manifest cancer would have developed if the experiment had been continued for 2 or 3 years.

Judging from these findings, the fact of aging may be involved in prostatic carcinogenesis, and chemical carcinogens alone may not be able to induce tumors at high incidence. We would suggest that the most effective procedure for inducing prostatic carcinogenesis might be cycles of administration of a chemical carcinogen after hormonal stimulation.

Further studies are necessary to develop a better system to induce prostate carcinoma at higher incidence in a shorter period. We are now planning another experiment in which testosterone and a chemical carcinogen will be administered repeatedly to ACI/Seg rats.

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