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Report

Effects of dehydroepiandrosterone and other sex steroid hormones on mammary carcinogenesis by direct injection of 7, 12-dimethylbenz(a) anthracene (DMBA) in hyperprolactinemic female rats

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Key words: adenocarcinoma, dehydroepiandrosterone, DMBA-induced mammary tumor, prolactin

Summary

The present study was performed to investigate the effects of dehydroepiandrosterone (DHEA) compared with those of sex steroid hormones on the mammary tumor induced by local injection of 7, 12-dimethylbenz(a) anthracene (DMBA) in hyperprolactinemic female rats. Under sustained hyperprolactinemia induced by pimozide (PMZ) from day 21, DMBA was injected locally into the mammary glandular tissues on day 73. Rats were divided into 5 groups as follows; steroid free (DP group), 17 β-estradiol (DP + E2 group), testosterone (DP + T group), progesterone (DP + Prog group), or dehydroepiandrosterone (DP + DHA group). The growth pattern and histological classification of the tumor in these 5 groups and rats treated only with DMBA (D group) were examined. All of the tumors grew to a size of 10 mm in diameter and after retaining the size for a certain duration, increased the size rapidly again (onset of rapid tumor growth). The period from the day of DMBA administration to that of onset of the rapid tumor growth in DP group was shorter than in D group, and the period in DP + DHA was longer than DP group and longest in steroid-treated groups. The incidence of adenocarcinoma was 2 tumors/16 animals in D group, 9/11 in DP group, 5/11 in DP + Prog group, 2/7 in DP + E2 group, 2/8 in DP + T group, and 0/10 in DP + DHA group. The incidence of adenocarcinoma in each steroid group except in DP + Prog group was lower than in DP group. These results suggest that prolactin (PRL) increases the incidence of adenocarcinoma in the DMBA-induced mammary tumor model, and DHEA especially decreases the incidence of adenocarcinoma.

Introduction

DMBA induced mammary tumor in rats is a widely used model of hormonal-sensitive breast cancer in women [1, 2]. This chemical carcinogen is usually given to the rat by oral or intravenous injection, but these models sometimes cause cancers in other organs and adrenal necrosis [3, 4]. To investigate the hormonal action on mammary epithelium at a peripheral site, these methods seemed to be not so useful. Although methods of direct injection of DMBA into mammary tissues had been designed [5], the locally applied carcinogen affects not only mammary epithelial cells but mesenchymal cells, resulting in a variety of histology [6].

It is well known that serum DHEA and dehydroepiandrosterone-sulfate (DHEA-S) decrease with age in women [7, 8]. The decline with age of serum levels of DHEA and DHEA-S has indicated that low serum DHEA and DHEA-S levels could

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be associated with breast cancer [9]. In order to investigate the peripherally preventive effect of DHEA on the development of mammary carcinoma, we designed a new method using direct injection of DMBA into mammary tissues in rats with continual treatment of pimozide, a dopaminergic blocker, for the induction of hyperprolactinemia beginning at day 21 to develop the mammary epithelial tissues for higher incidence of adenocarcinoma. Using this model, the effect of DHEA on the occurrence of mammary tumors was compared with that of other sex steroids: 17 β-estradiol (E2), testosterone (T), and progesterone (Prog).

Materials and methods

Animals

Immature Wistar female rats (Charles River Japan Inc., Atugi) on the 21st day were housed in group cages (two or three rats per cage) under standard conditions of light (lights on from 0500–1900 h) and temperature (21–25° C).

Treatment

Pimozide (Fujisawa Co., Osaka) was dissolved in the drinking water (from day 21); it was first dissolved in 50 ml 0.1 M tartaric acid (Wako Junyaku, Osaka) and then diluted with tap water, to a concentration of 10 mg/liter. The pimozide solution always contained 1% glucose (Wako Junyaku) to make it palatable to the animals. Untreated animals drank the proper control solutions. After 69 Wistar female rats on day 21 were divided into a pimozide-treated group (53 rats) and a pimozide-untreated group (16 rats), the treatment of pimozide or control solutions was started. On day 73, all these rats were anesthetized with ether and a small incision was made in the right inguinal mammary gland of each rat. The mammary tissue was exposed and a 4 mg single dose of DMBA (Wako Junyaku) dissolved in 0.1 ml of sesame oil (Hanni Chem. Co., Kyoto) was injected into it with 27 G needle and the skin was closed with Mitchell. After DMBA administration, the pimozide-treated rats were divided into 5 groups as follows:

1) no steroid-treated group (n = 11),
2) DHEA-treated group (n = 10); DHEA acetate (Teikoku Zouki Co., Tokyo) 50 mg/ml, 5 mg/100 g BW, 3 times a week,
3) E2-treated group (n = 11); Ovahormon depot (Teikoku Zouki Co.), 17 β-estradiol dipropionate, 5 mg/ml, 1 mg/100 g BW, 1 time a week,
4) T-treated group (n = 10); Enarmon depot (Teikoku Zouki Co.), testosterone enanthate 125 mg/ml, 6 mg/100 g, 1 time a week, and
5) Prog-treated group (n = 11); Luteum (Teikoku Zouki Co.), progesterone 10 mg/ml, 1 mg/100 g BW, 3 times a week.

These steroids were all dissolved in sesame oil and subcutaneously injected. Rats in a pimozide-untreated (only DMBA-treated) group were not treated with any steroid. Each mammary tumor was measured three times a week with calipers to the nearest mm for length and width. The mean of these measurements was determined for each tumor. Each stage of tumor growth was determined as follows; the day of DMBA administration (day 0), the period of the beginning of rapid tumor growth again after remaining same size for some period (onset of rapid tumor growth). When the sizes of these tumors reached 50 mm, or 30 mm if the tumor had an ulcer, rats were killed by decapitation, and histological examination followed. Treatment of pimozide and each one of the hormones continued until the rats were killed.

Calculations

Statistical significance was calculated according to two sample t test or two sample t test with Welch's correction. Analysis of differences in the occurrence of adenocarcinomas was performed using the chi-square and Fisher's exact frequency tests.

Result

1. The histological changes of mammary tissues in rats treated with pimozide:

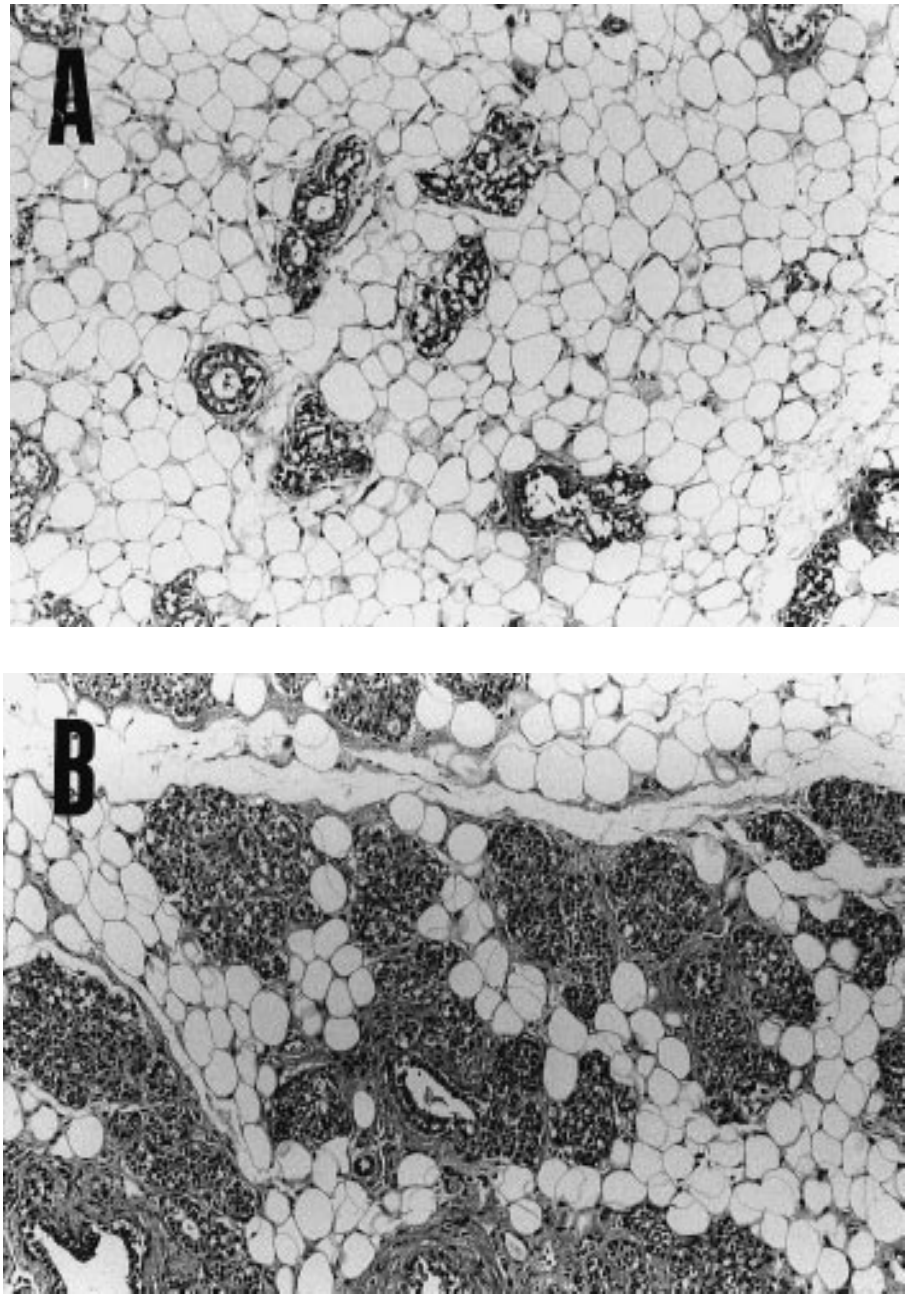

Figure 1. Light microscopic findings of the mammary tissue of control rat and pimozide-treated rat on day 37. (A) The control rat. Poorly developed alveolar cells are seen. × 168. H.E. stain. (B) The pimozide-treated rat. There are markedly developed alveolar cells. × 168. H.E. stain.

1) Microscopic finding of mammary tissues treated 16 days with PMZ (day 37):
In pimozide-untreated group, poorly developed alveolar cells were seen in the adipose tissue (Figure 1A). In pimozide-treated group, there was marked proliferation of the glandular tissue, with an increase both in the number of lobules and in the number of acini with each lobule. The acinar cells had slightly vacuolated cytoplasm (Figure 1B).
2) The mammary glandular tissues treated 52 days

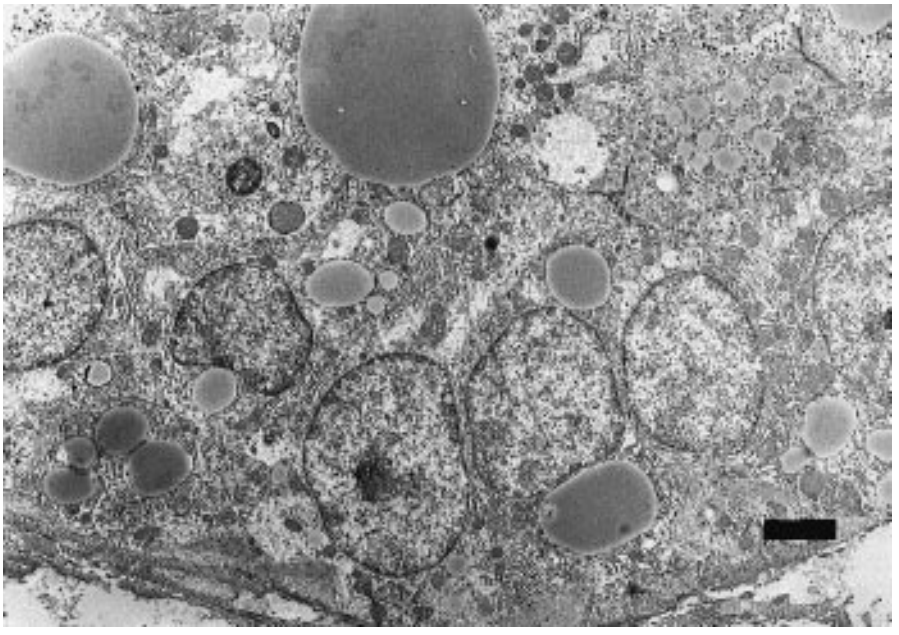

Figure 2. Electron micrograph of the mammary gland of pimozide-treated rat on day 73. There are many lipid droplets in the cytoplasm. Scale bar indicates 2 μm.

with PMZ (day 73): The acini were dilated and contained luminal secretion. In electron microscopic findings of acini of PMZ group, there were many lipid droplets and secretory vesicles in active epithelial cells (Figure 2).

2) *Growth of DMBA – induced mammary tumors:* The appearance of DMBA-induced mammary tumors was recognized in all groups. These tumors developed only in the right inguinal mammary gland where DMBA was applied, and were not observed in any other gland. The comparison with mean tumor diameter in each group was followed. The diameter in DP group was significantly larger than in D group 30 days after DMBA injection (Figure 3). In DP + E2 group, in DP + T group, and in DP + DHA, all diameters of tumors were smaller than in DP group 70–80 days after DMBA injection. In DP + Prog, the diameters of tumors were also smaller than in DP group 70–80 days, but 110 days after DMBA injection, there was no difference from the DP group (Figure 4). The onset of rapid tumor growth in D group and in DP group was 85.2 ± 28.7 (mean ± S.D.) and 61.8 ± 20.2 days, respectively, which means that pimozide treatment shortened the period to onset of rapid tumor growth. The period of onset of rapid tumor growth in the steroid-treated groups was 118 ± 16.8 days in DP + DHA group, 99.9 ± 23.3 in DP + E2 group, and 75.1 ± 27.1 days in DP + T group, which means

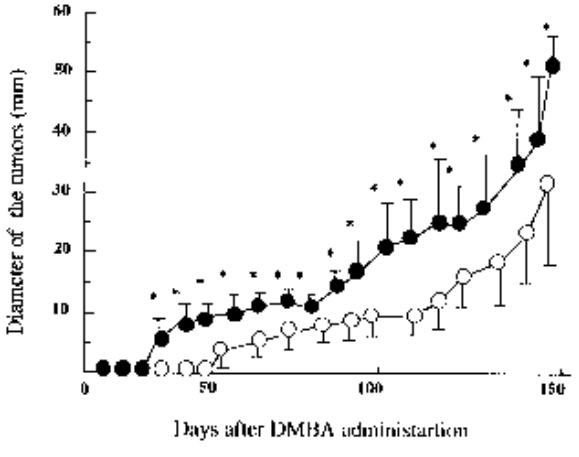

Figure 3. Diameters of tumors in DMBA + pimozide (DP) group and DMBA (D) group during 150 day period after DMBA administration. The diameter of tumors in DP group was larger than that in D group on 30 days after DMBA administration. (○), DP group; (●), D group. * $p < 0.05$ vs D group. Bars represent mean ± S.D.

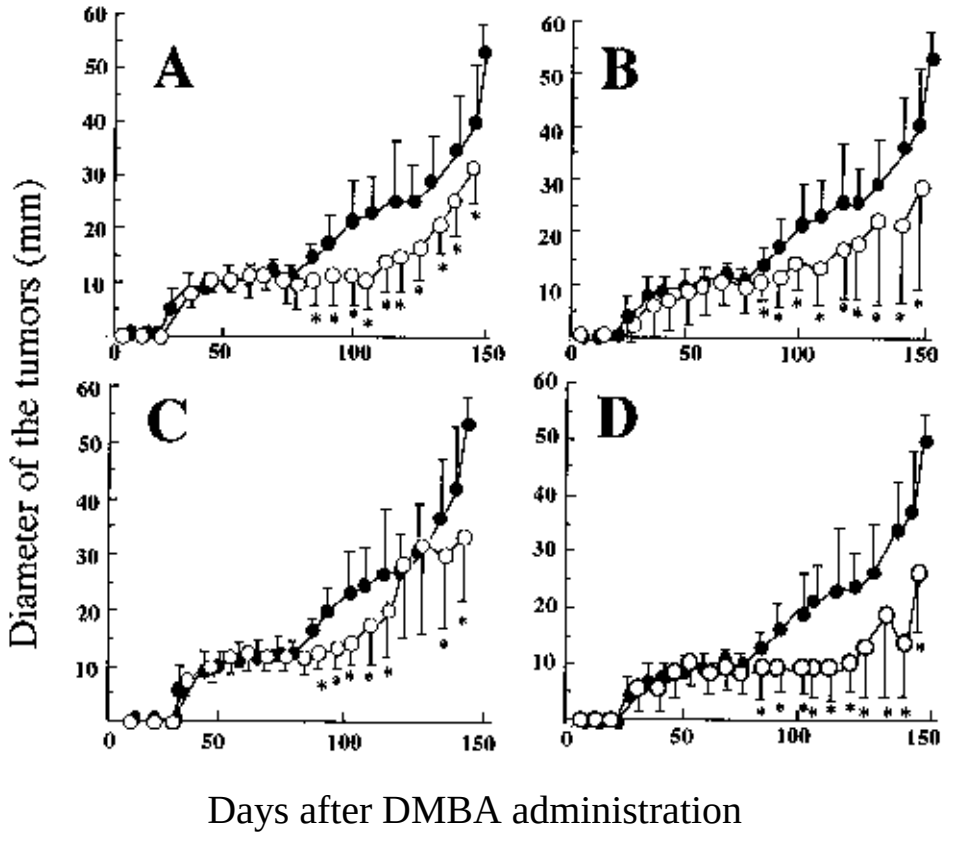

Figure 4. The effects of steroid on the growth of tumors in steroid-treated groups during 150 day period after DMBA administration. After the onset of rapid tumor growth, the diameters of tumors in all steroid groups were smaller than that in DP group. A, DP + 17β estradiol group; B, DP + testosterone group; C, DP + progesterone group; D, DP + dehydroepiandrosterone-acetate group.
C, DP group; (C), steroid-treated groups. * p AN 0.05 vs DP group. Bars represent mean ± S.D.

that steroid treatment on DMBA-induced mammary tumors treated with pimozide shortened the period to onset of rapid tumor growth. In comparison with the period of each of the steroid-treated groups, the period in DP + DHA group was significantly longer than in any other steroid-treated groups (Figure 5). In DP + E2 and DP + T group, ulcer active changes were observed in some tumors when they grew to more than 30 mm in diameter. And in all of rats in this experiment, no rats had died due to the growth of DMBA induced mammary tumor except 4 out of 11 rats in DP + T group which died from other reasons.

3. *The histological types in DMBA induced mammary tumors:*

All of the histological types of DMBA-induced mammary tumors were shown in Table 1. WHO criteria were applied for classifying each tumor type. Adenocarcinomas appeared as ductal carcinoma (Figure 6A) and papillary carcinoma. The microscopic appearance of adenocarcinomas did not seem to be influenced by each steroid treatment. In sarcomas, spindle shaped cells of various sizes were seen in fascicular pattern (Figure 6B).

The respective incidence of adenocarcinoma in D group and DP group was 13% and 82%, which indicated that there was significant increase of the incidence in DP group. The incidence of adenocarcinoma in the steroid-treated group was 46% in DP + Prog group, 28% in DP + E2 group, 25% in DP + T group, and 0% in DP + DHA group, which showed the incidence in each steroid treated group except in DP + Prog group was significantly lower

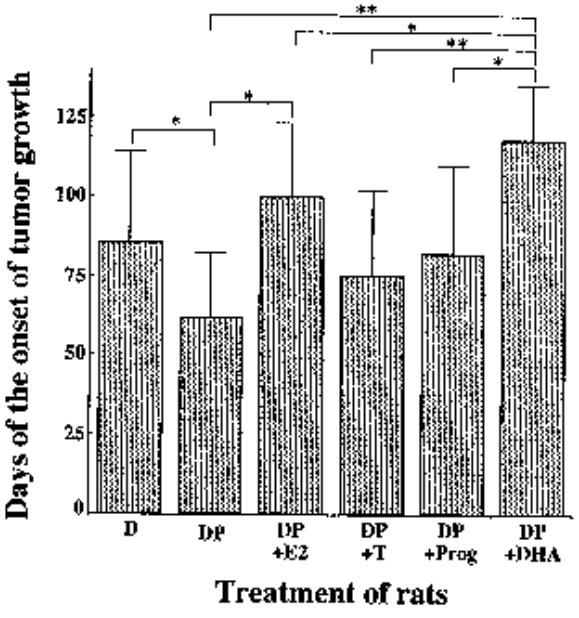

Figure 5. Period from the day of DMBA administration to the day of onset of tumor growth in DMBA-induced rat mammary tumors. The period in DP group was 23 days shorter than that in DMBA group. On the other hand, the period in DP + dehydroepiandrosterone group was 51 days longer than that in DP group. DP, DMBA + pimozide; DP + E2, DP + 17β estradiol; DP + T, DP + testosterone; DP + Prog, DP + progesterone; DP + DHA, DP + dehydroepiandrosterone acetate. * $p < 0.05$, ** $p < 0.01$. Bars represent mean ± S.D.

than in DP group, and the incidence in DP + DHA group was lower than in DP + Prog group (Figure 7).

Discussion

DMBA is very well known to be a carcinogenic agent for mammary tumors by oral injection to stomach [10], but it sometimes causes leukemia, lung cancer, ovarian cancer, and adrenal necrosis at the same time, due to the carcinogenic effect doing harm to all internal organs [11, 12]. Induction of selective mammary tumor with DMBA by oral injection without any influence on other organs has been said to be difficult, especially in Wistar rats [13].

To investigate the mechanism of the hormonal inhibitory effect on DMBA-induced mammary tumors, it is important for the carcinogen to act directly in mammary tissues because the steroid hormone has the possibility of acting on areas outside the breast. DMBA is chiefly metabolized in the liver [5] by oral or intravenous administration. If DHEA has modified the hepatic metabolism [14, 15], more carcinogen would be converted to inactive metabolites. Therefore, less active DMBA could reach the target tissue, resulting in a decrease of cancer incidence. For the solution of these problems, DMBA was dusted directly onto the mammary gland [16] instead of feeding the carcinogen into the stomach. Terada et al. selectively induced ductal adenocarcinoma by intraductally injecting DMBA into one mammary gland of postpartum Wistar inbred rats [17], indicating that the long-term direct effect of residual DMBA on the epithelioglandular cells was important for inducing adenocarcinoma. In our experiment, the carcinogen was first dissolved in se-

Table 1. The histological types of DMBA-induced mammary tumors under the administration of pimozide and several steroids

DMBA group[a]	Number of rats	Number of rats bearing tumor with the histological types of							
		Adeno-carcinoma	Adenocarcinoma + sarcoma	Fibroadenoma + sarcoma	Hyperplasia	Sarcoma	Fibrosarcoma	Fibroma	Fibroma + sarcoma
D	16	2 (12.5%)	N.D.[b]	1 (6.3%)	1 (6.3%)	6 (37.4%)	4 (25.0%)	2 (12.5%)	N.D.
DP	11	8 (72.7%)	1 (9.1%)	N.D.	1 (9.1%)	1 (9.1%)	N.D.	N.D.	N.D.
DP + E2	7	1 (14.3%)	1 (14.3%)	N.D.	N.D.	4 (57.1%)	N.D.	1 (14.3%)	N.D.
DP + T	8	2 (25.0%)	N.D.	N.D.	N.D.	2 (25.0%)	2 (25.0%)	2 (25.0%)	N.D.
DP + Prog	11	5 (45.5%)	N.D.	N.D.	N.D.	4 (36.3%)	2 (18.2%)	N.D.	N.D.
DP + DHA	10	N.D.	N.D.	N.D.	N.D.	4 (40.0%)	2 (20.0%)	3 (30.0%)	1 (10.0%)

a) D, DMBA; DP, D + pimozide; DP + E2, DP + 17β-estradiol; DP + T, DP + testosterone; DP + Prog, DP + progesterone; DP + DHA, DP + dehydroepiandrosterone-acetate.
b) N.D. not detected.

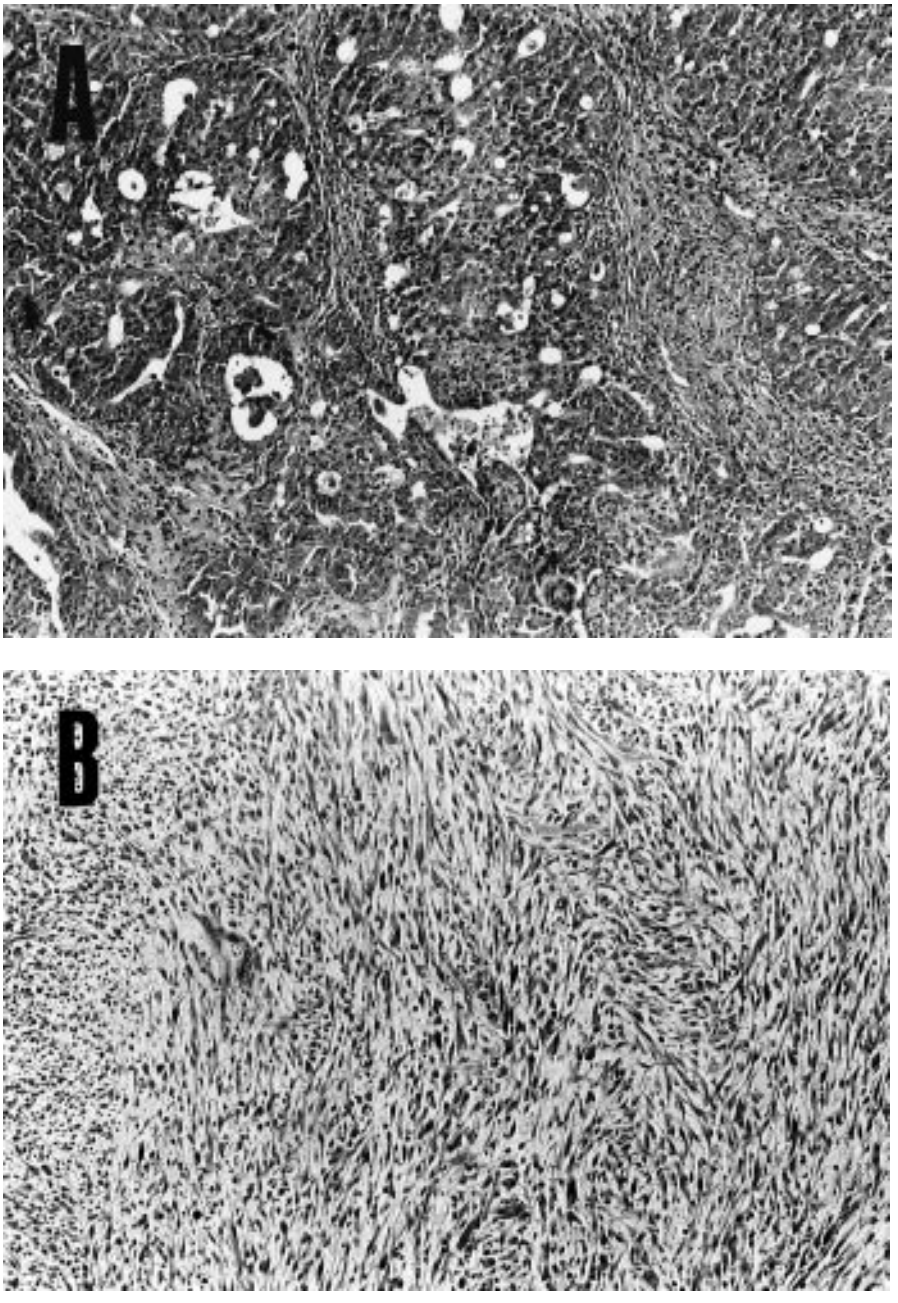

Figure 6. Typical light microscopic findings of DMBA-induced mammary tumor. (A) Invasive ductal carcinoma of the mammary tumor. × 168. H.E. stain. (B) Sarcoma of the mammary tumor. Note ductal pattern in (A) and spindle shaped tumor cells in (B). × 168. H.E. stain.

same oil, which might aid diffusion into the normal mammary tissues where lipid cells are predominant (refer to Figure 1), resulting in high occurrence of mammary tumors (16 tumors/16 rats in D group) compared with the 'dusting' method previously mentioned [16]. Because of the structural features in resting mammary tissues, a small number of glandular cells surrounded with many lipid cells, the carcinogen might stimulate mainly lipid or other stromal cells, resulting in very low occurrence of adenocarcinoma. Under chronic hyperprolactinemia induced by PMZ, the glandular tissue showed an

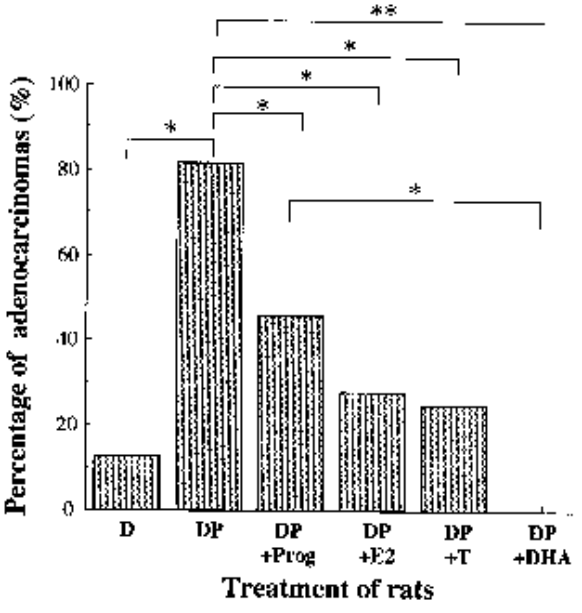

Figure 7. Percentage of adenocarcinomas in each group. The percentage of adenocarcinomas was reduced by steroid treatment, especially by dehydroepiandrosterone-acetate injection. D, DMBA; DP, D + pimozide; DP + E2, DP + 17β estradiol; DP + T, DP + testosterone; DP + Prog, DP + progesterone; DP + DHA, DP + dehydroepiandrosterone-acetate. * $p < 0.05$; ** $p < 0.01$.

increase both in the number of lobules and in the number of acini within each lobule, containing lipid droplets and secretory vesicles in active epithelial cells. These developments seemed to be extremely useful for retaining DMBA dissolved in sesame oil for a long term in the mammary epithelial tissues and in the cytoplasm of epithelial cells. With regard to inducing hyperprolactinemia, Advis et al. [18] reported that in rats treated since day 21 with pimozide, which is a dopaminargic receptor blocker, the hyperprolactinemic state was kept on with high levels of endogenous E2 and Prog. The endocrinological environment induced by PMZ led the epithelial tissues to the proliferative state shown in Figure 1B and Figure 2. The incidence of adenocarcinoma in the pimozide-treated group was significantly higher (82%) than that in the pimozide-untreated group (15%), and there was no metastasis and no tumor appearance in other organs in the rats after treatment with DMBA, indicating that our method in this experiment is very useful for specifically inducing mammary adenocarcinomas in Wistar female rats.

The tumor growth represented in Figure 4 shows that there was no significant difference, compared with DP group, during the period from the DMBA administration to the onset of rapid tumor growth, and after the period, the tumor growth in each steroid-treated group was suppressed, which indicates that the period of the onset of rapid tumor growth is very important for detecting the suppressive effect of each steroid on tumor growth in this experiment. E2, T, and Prog in this experiment prolonged the onset of rapid tumor growth and (except progesterone) reduced the incidence of adenocarcinoma, which means that these steroid hormones have the potential to reduce the progression of mammary adenocarcinoma. This mechanism has been recognized in the reduction of PRL receptor and estrogen receptor of the adenocarcinomas [19–23].

DHEA has been shown to protect against chemical carcinogenesis [24] and spontaneous appearance of mammary cancer in mice [25]. In humans, a low urinary excretion of DHEA metabolites has been found in mammary cancer patients [26, 27] and in normal patients who subsequently developed breast cancer [28, 29]. Also DHEA sulfate concentrations were found to be lower in the cytosol of malignant than in nonmalignant human breast tissue [30, 31]. And as regards rats, Boccuzzi et al. [32] described that DHEA reduced the growth of DMBA-induced mammary tumors in intact rats.

The present study clearly demonstrates that in pimozide-treated rats the appearance of adenocarcinoma is extremely reduced by DHEA injection. The explanation for this finding is not obvious. Recently, DHEA has been reported to antagonize E2 on the growth of a human breast cancer cell line, by an interaction between its metabolite ADIL and estrogen receptor [33]. Inhibition of tumor growth through a DHEA-androgen receptor (AR) interaction [34] is also important, since the activation of AR prevents DMBA-induced mammary tumor growth in rats [35]. It is well recognized that DHEA is a noncompetitive inhibitor of glucose-6-phosphate-dehydrogenase (G6PD) [36, 37]. Compared with other sex steroids, E2, T, or Prog, the inhibition of epidermis G6PD was most observed in DHEA-treated mice, and DHEA markedly inhibited DNA-synthesis in breast epithelium, as did 16α-Br-

epiandrosterone which is more active than DHEA as an inhibitor of G6PD [38]. Referring to the doses of steroids used in the above experiment, we selected the doses of E2, T, Prog, or DHEA per body weight in our study. Down regulation of PRL should be considered as a mechanism of the reduction of adenocarcinoma in our experiments [39, 40]. Recently Yan et al. [41] studied the effect of increasing serum levels of DHEA released from Silastic implants on the incidence of these tumors in the rat. Treatment with increasing doses of DHEA caused a progressive inhibition of tumor development with increased levels of PRL, which indicates that the preventive effect of DHEA on mammary carcinoma in our experiment is not due to the reduction of PRL. About the result of the onset of rapid tumor growth and the incidence of adenocarcinoma in this experiment, Daniel and Prichard [42] have observed that adenocarcinomas tend to arise earlier than other histological types of tumors following intragastric intubation of DMBA. There seems to be a similar association between the onset of rapid tumor growth and the incidence of adenocarcinoma in this study. As regards the effect of these steroids on stromal components, like lipid cells and fibroblasts, stimulus or inhibition of E on fibroblast growth was investigated [43, 44] and the stimulating effect of T on fibroblast proliferation was also reported [45, 46], which indicates the possibility of increasing the sensitivity for DMBA, resulting in the small incidence of sarcomas by E or T treatment in our study. Regarding DHEA, Saenger et al. studied the inhibitory action on fibroblast growth [47], and Shantz et al. demonstrated the suppression of growth and differentiation in 3T3-L1 mouse embryo fibroblasts [48], which indicates that the incidence in our experiment of nonepithelial tumors, which are mainly malignant, may be due to the suppression of the susceptibility of epithelial cells to DMBA caused by treatment with DHEA, rather than to increase of the sensitivity of stromal components to the carcinogen.

Although its mechanism of inhibition of mammary carcinoma in this experiment is still unclear, DHEA acts on the peripheral site to protect against the occurrence of mammary adenocarcinoma induced by direct injection of DMBA.

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