Effects of Various Doses of 17 β -estradiol on the Progression of Mammary Carcinomas Induced by Single and Multiple Administration of 7, 12-Dimethylbenz[a]anthracene in Female Rats

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Abstract: The objective of this investigation was to elucidate the role of sex hormones in the progression and subsequent hormone dependency of mammary carcinomas induced by single or multiple administration of 7, 12-dimethylbenz[a]anthracene (DMBA). Ten milligrams of DMBA were administered orally to female Sprague-Dawley (SD) rats once at the age of 42 days (Group A) and three times at the ages of 28, 42 and 56 days (Group B). All 70-day-old rats in Groups A and B were divided into 4 groups (I, II, III and IV), respectively. Rats in Groups I of A and B were intact controls. Rats in Groups II of A and B were ovariectomized (OVEX) at the age of 70 days. Rats in Group II of Group A and in Groups III of Groups A and B were divided into 4 subgroups (Group II-1, II-2, II-3 and II-4) and 3 subgroups (Group III-1, III-2 and III-3), respectively. Rats in Groups II-2 of A and III-1 of A and B, rats in Groups II-3 of A and III-2 of A and B and rats in Groups II-4 of A and III-3 of A and B were given injections of 0.01, 0.1 and 1 mg 17 β -estradiol (E₂), respectively. Rats in Groups IV of A and B were given injections of 4 mg progesterone (P). Injections of E₂ or P were given 3 times a week between the ages of 70 and 217 days in Group A and the ages of 70 and 105 days in Group B. In Group A, there was no difference of incidence of mammary carcinomas among Groups I, II-2, II-4, III-1 and IV but the incidence of mammary carcinomas in Groups II-1, II-3, III-2 and III-3 was extremely low, compared with that in Group I. In Group B, a large number of mammary carcinomas developed and although there was no difference in the incidence of mammary carcinomas among Groups I, II, III and IV, the number of mammary carcinomas per rat decreased in Groups II, III-2 and III-3, compared with Group I. These findings indicate that the progression of mammary carcinomas induced by a single or multiple administration of DMBA was suppressed in ovariectomized rats and in rats with injections of high doses of E₂. DMBA-induced mammary carcinomas in Group B were transplanted into OVEX rats (R-1), OVEX rats with injections of 0.01 mg E₂ (R-2) and OVEX rats with injections of 1 mg E₂ (R-3). According to tumorigenesis in those rats with various hormonal treatments (R-1, 2, 3), mammary carcinomas were classified into one of the four categories (Type A, B, C and D). Type A tumor: tumor developed greatly in R-2 but slightly or not in R-1 and R-3. Type B tumor: tumors developed greatly in R-1 but slightly or not in R-2 and R-3. Type C tumor: tumors developed greatly in R-3 but slightly or not in R-1 and R-2. Type D tumor: tumors developed greatly in R-1, R-2 and R-3. In Group B, rates of type A, B, C, D tumor were 43.2, 13.5, 24.3 and 18.9% in Group I, 26.3, 10.5, 21.1 and 42.1% in Group II, 40.0, 5.0, 45.0 and 10.0% in Group III-1, 38.5, 15.4, 30.8 and 15.4% in Group III-2, 28.6, 0, 71.4 and 0% in Group III-3 and 44.4, 33.3, 11.1 and 11.1 in Group IV, respectively.

These results suggested that the hormonal status of the development process may be one of factors which affects the characteristics of hormone dependency and the multiple administration of DMBA may induce large numbers of carcinoma cells with varying dependency on hormones and carcinoma cells may be selected in hormonal conditions during progression. (*J Toxicol Pathol* 2000; 13: 65~75)

Key words: mammary carcinoma, 7, 12-dimethylbenz[a]anthracene, rat, 17 β -estradiol, progesterone, progression

Introduction

The development and growth of mammary carcinomas induced in female rats by a single administration of 20 mg 7, 12-dimethylbenz[a]anthracene (DMBA) are dependent on the present of hormones, particularly ovarian hormones and

prolactin^{1,2}, but some DMBA-induced mammary carcinomas are estrogen-independent as indicated by continued growth after ovariectomy. It has been reported that the first and/or second week after carcinogen administration to rats is critical to the development of mammary carcinomas and that suppression of the secretion of estrogen and/or prolactin during this period results in permanent inhibition of mammary carcinogenesis³⁻⁵. The purpose of this study was to determine the roles of sex hormones, particularly various doses of estrogen on the progression and subsequent hormone dependency of mammary carcinomas induced by single or multiple administration of DMBA.

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Material and Methods

Animals

All the animals were Inbred Sprague-Dawley (SD) female rats, maintained in a filtered air laminar flow at the Faculty of Medicine of Kagoshima University. The animals were given a commercial diet (CE-2, CLEA Inc., Tokyo Japan) and tap water was available *ad libitum*. The room temperature was maintained at $23 \pm 2^\circ\text{C}$ and the relative humidity, at $55 \pm 10\%$, with a 12 hr-light/dark cycle. This study was carried out in accordance with Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

Induction of mammary carcinomas and hormonal treatment

Ten milligrams 7, 12-dimethylbenz(a)anthracene (DMBA,

Wako Pure Chemical Industries Ltd., Osaka Japan) dissolved in 1 ml sesame oil were orally administered to 93 SD female rats once at the age of 42 days (Group A, **Table 1**), or to 138 SD female rats 3 times at the ages of 28, 42 and 56 days (Group B, **Table 3**). All 70-day-old rats in Groups A and B were sub-divided into 4 groups (I, II, III, IV). Rats in Groups I and IV of A and B were given injections of 0.05 ml vehicle (mixed solution of saline, 0.4% Tween 80, 0.5% carboxymethylcellulose and 0.9% benzyl alcohol) and 4 mg progesterone (P, Sigma chemical Co., St. Louis USA) in 0.05 ml vehicle, respectively. Rats in Groups II of A and B were ovariectomized at the ages of 70 days. Rats in Group II of A were divided into 4 subgroups (Groups II-1, II-2, II-3, II-4). Rats in Groups III of A and B were divided into 3 subgroups (Groups III-1, III-2, III-3). Rats in Groups II-2 of A and III-1 of A and B, rats in Groups II-3 of A and III-2 of A and B and rats in Groups II-4 of A and III-3 of A and B were given injections of 0.01, 0.1 and 1 mg 17β -estradiol (E_2 ,

Table 1. Incidence of Mammary Carcinomas in Inbred SD Female Rats Receiving Hormonal Treatment and A Single Administration of DMBA

Group		Age (days)	No. of rats examined	No. of rats with tumors	No. of tumors per group	No. of tumors per rat	Latent period (days)
I	Intact	119	12	3 (25.0%)	6	0.5 ± 1.00	106.40 ± 44.52
		168	11	5 (45.5%)	7	0.6 ± 0.81	
		217	10	6 (60.0%)	10	1.0 ± 1.05	
II-1	Ovariectomy (Intact)	119	17	0 (0.0%)	0	0.0 ± 0.00	0.00 ± 0.00
		168	17	0 (0.0%)**	0	0.0 ± 0.00	
		217	17	0 (0.0%)**	0	0.0 ± 0.00	
II-2	Ovariectomy 17β -estradiol (0.01 mg/body)	119	5	0 (0.0%)	0	0.0 ± 0.00	121.33 ± 26.50
		168	5	1 (20.0%)	1	0.2 ± 0.45	
		217	5	2 (40.0%)	3	0.6 ± 0.89	
II-3	Ovariectomy 17β -estradiol (0.1 mg/bod)	119	5	0 (0.0%)	0	0.0 ± 0.00	0.00 ± 0.00
		168	5	0 (0.0%)	0	0.0 ± 0.00	
		217	5	0 (0.0%)*	0	0.0 ± 0.00	
II-4	Ovariectomy 17β -estradiol (1 mg/body)	119	5	0 (0.0%)	0	0.0 ± 0.00	0.00 ± 0.00
		168	5	0 (0.0%)	0	0.0 ± 0.00	
		217	2	0 (0.0%)	0	0.0 ± 0.00	
III-1	17β -estradiol (0.01 mg/body)	119	12	1 (8.3%)	1	0.1 ± 0.29	132.50 ± 34.89
		168	12	3 (25.0%)	4	0.3 ± 0.65	
		217	9	6 (66.7%)	9	1.0 ± 0.87	
III-2	17β -estradiol (0.1 mg/body)	119	12	0 (0.0%)	0	0.0 ± 0.00	136.50 ± 14.80
		168	12	1 (8.3%)	1	0.1 ± 0.29	
		217	12	1 (8.3%)*	2	0.2 ± 0.58	
III-3	17β -estradiol (1 mg/body)	119	13	0 (0.0%)	0	0.0 ± 0.00	0.00 ± 0.00
		168	13	0 (0.0%)*	0	0.0 ± 0.00	
		217	10	0 (0.0%)**	0	0.0 ± 0.00	
IV	Progesterone (4 mg/body)	119	12	3 (25.0%)	4	0.3 ± 0.65	113.56 ± 56.41
		168	12	4 (33.3%)	5	0.4 ± 0.67	
		217	11	4 (36.4%)	5	0.5 ± 0.69	

Ten mg DMBA were orally administered to 93 female rats once at age 42 days. Ovariectomy was performed 4 weeks after administration of DMBA and 17β -estradiol or progesterone was given 3 times a week, starting from 4 weeks after administration of DMBA. Each value represents the mean $\pm$ S.D..

* $p < 0.05$; ** $p < 0.01$: significantly different from Group I

Sigma Chemical Co., St. Louis USA), respectively, in 0.05 ml vehicle. Injections were given 3 times a week between the ages of 70 and 217 days in Group A and between 70 and 105 days in Group B. All rats were examined by palpation to detect mammary tumors twice a week until the age of 217 days in Group A and the age of 105 days in Group B. All DMBA-induced mammary tumors were excised by the age of 217 days in Group A and the age of 105 days in Group B, fixed in 10% phosphate-buffered formalin, dehydrated, embedded in paraffin wax. Widest cut surfaces were sectioned to 5 μ m, stained with H.E. stain and examined histologically. Mean differences were evaluated by the t-test. The incidence of tumors was tested by a fourfold contingency table⁶.

Transplantation

Mammary carcinomas in Group B were excised from the rat and minced. Approximately 80 to 100 mg of the minced carcinoma tissue were transplanted at the upper and middle milk lines of 70-day-old ovariectomized rats (R-1), 70-day-old ovariectomized rats with injections of 0.01 mg E₂ (R-2) and 70-day-old ovariectomized rats with injections of 1 mg E₂ (R-3). Ovariectomy was done and injections started on the day of transplantation. Transplanted carcinomas in rats were measured on their major and minor axes by palpation twice a week for 8 weeks.

Immunohistochemical staining for prolactin

Pituitaries were fixed in 10% phosphate-buffered formalin (PBF) at room temperature and embedded in paraffin. Sections were cut from paraffin embedded tissue blocks, deparaffinized and rehydrated. They were subjected to the

standard avidin-biotin complex immunoperoxidase assays using the Vectastain ABC kit (Vector Laboratories, USA). Deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol solution to block endogenous peroxidase activity. They were placed in phosphate-buffered saline (PBS), and were subsequently incubated at room temperature with the following reagents (with PBS washes in between): 1.0% normal bovine serum albumin (BSA)-PBS-NP40 for 30 min. for blocking, primary antibody, prediluted rabbit anti-human prolactin (DAKO CORPORATION, USA) for 60 min., biotinylated goat anti-rabbit immunoglobulin (IgG) for 60 min., ABC complex for 60 min., and 3,3'-diaminobenzidine tetrachloride (DAB) containing 0.05% hydrogen peroxide for 5 min. The sections were then washed, counter-stained, dehydrated, cleared in xylene, and mounted.

Results

Incidences of mammary carcinomas in Group A are shown in Table 1.

There was no difference in incidence of mammary carcinoma among Groups I, II-2, II-4, III-1 and IV. Incidences of mammary carcinomas in 168-day-old rats and 217-day-old rats of Group II-1, 217-day-old rats of Group II-3, 217-day-old rats of Group III-2 and 168-day-old rats and 217-day-old rats of Group III-3 were extremely low, compared with those of Group I. The latent period of the above-described tumors ranged from 106.40 days to 136.50 days, showing no statistically significant difference.

Table 2. Absolute and Relative Organ Weights in Inbred SD Female Rats Receiving Hormonal Treatment and A Single Administration of DMBA

Group		Absolute organ weights			Relative organ weights		
		Pituitary (mg)	Adrenals (mg)	Ovaries (mg)	Pituitary (mg/g)	Adrenals (mg/g)	Ovaries (mg/g)
I	Intact	139.1 $\pm$ 15.64	375.6 $\pm$ 61.59	836.7 $\pm$ 175.36	0.49 $\pm$ 0.056	1.31 $\pm$ 0.161	2.91 $\pm$ 0.523
II-1	Ovariectomy (Intact)	118.1 $\pm$ 14.50**	271.3 $\pm$ 25.90**	—	0.35 $\pm$ 0.044**	0.80 $\pm$ 0.067**	—
II-2	Ovariectomy 17 β -estradiol (0.01 mg/body)	355.0 $\pm$ 58.00**	437.8 $\pm$ 55.27	—	1.32 $\pm$ 0.212**	1.62 $\pm$ 0.121**	—
II-3	Ovariectomy 17 β -estradiol (0.1 mg/body)	786.4 $\pm$ 179.46**	335.4 $\pm$ 58.56	—	3.53 $\pm$ 0.718**	1.51 $\pm$ 0.253*	—
II-4	Ovariectomy 17 β -estradiol (1 mg/body)	1070.3 $\pm$ 379.23**	382.3 $\pm$ 74.81	—	5.95 $\pm$ 2.494**	2.08 $\pm$ 0.539**	—
III-1	17 β -estradiol (0.01 mg/body)	446.4 $\pm$ 152.97**	372.1 $\pm$ 70.66	456.4 $\pm$ 187.57**	1.61 $\pm$ 0.531**	1.36 $\pm$ 0.311	1.68 $\pm$ 0.735**
III-2	17 β -estradiol (0.1 mg/body)	808.9 $\pm$ 181.69**	303.3 $\pm$ 32.86**	455.5 $\pm$ 92.87**	3.85 $\pm$ 1.011**	1.44 $\pm$ 0.147	2.17 $\pm$ 0.538**
III-3	17 β -estradiol (1 mg/body)	1018.3 $\pm$ 466.62**	351.1 $\pm$ 45.42	376.7 $\pm$ 112.13**	5.82 $\pm$ 3.339**	1.97 $\pm$ 0.457**	2.11 $\pm$ 0.465**
IV	Progesterone (4 mg/body)	229.2 $\pm$ 75.28**	408.8 $\pm$ 44.31	474.3 $\pm$ 161.17**	0.73 $\pm$ 0.269*	1.29 $\pm$ 0.138	1.50 $\pm$ 0.548**

Ten mg DMBA were orally administered to 93 female rats once at age 42 days. Ovariectomy was performed 4 weeks after administration of DMBA and 17 β -estradiol or progesterone was given 3 times a week, starting from 4 weeks after administration of DMBA. Each value represents mean $\pm$ S.D..

* $p < 0.05$; ** $p < 0.01$: significantly different from Group I

The weights of the organs of rats in Group A are shown in Table 2.

Absolute and relative weights of the pituitary in Group II-1 decreased significantly, compared with those in Group I. Conversely in Groups II-2, II-3, II-4, III-1, III-2, III-3 and IV, these weights increased significantly, compared with those in Group I. Absolute weights of the adrenal glands in Group II-1 and in Group III-2 decreased significantly, compared with those in Group I. However, the relative weights of adrenal glands in Group II-1 decreased significantly but those in Groups II-2, II-3, II-4 and III-3 increased significantly, compared with those in Group I.

Absolute and relative weights of the ovaries in Groups III-1, III-2, III-3 and IV decreased significantly, compared with those in Group I.

Incidences of mammary carcinomas in Group B are shown in Table 3.

A multiple administration of DMBA induced a large number of mammary carcinomas in all groups. The number of tumors in 84-day-old rats and 105-day-old rats of Groups II, III-2 and III-3 decreased significantly, compared with that in Group I. Latent periods of mammary carcinomas in Groups II and III-3 became protracted, compared with those in Group I.

Table 3. Incidence of Mammary Carcinomas in Inbred SD Female Rats Receiving Hormonal Treatment and A Multiple Administration of DMBA

Group	Age (days)	No. of rats examined	No. of rats with tumors	No. of tumors per group	No. of tumors per rat	Latent period (days)
I Intact	84	27	24 (88.9%)	109	4.0±2.56	
	105	19	19 (100.0%)	183	9.6±3.79	62.69±10.51
II Ovariectomy (Intact)	84	26	11 (42.3%)**	12	0.5±0.58**	
	105	26	21 (80.8%)	48	1.8±1.79**	71.06±13.52**
III-1 17 β -estradiol (0.01 mg/body)	84	17	17 (100.0%)	75	4.4±2.24	
	105	5	5 (100.0%)	41	8.2±2.59	59.09±8.53
III-2 17 β -estradiol (0.1 mg/body)	84	17	14 (82.4%)	39	2.3±2.31*	
	105	14	13 (92.9%)	62	4.4±3.08**	63.39±12.96
III-3 17 β -estradiol (1 mg/body)	84	26	17 (65.4%)*	38	1.5±1.63**	
	105	23	21 (91.3%)	75	3.2±3.19**	66.14±14.72*
IV Progesterone (4 mg/body)	84	25	25 (100.0%)	113	4.5±2.99	
	105	12	12 (100.0%)	100	8.3±4.98	61.05±10.49

Ten mg DMBA were orally administered to 138 female rats 3 times at ages 28, 42 and 56 days. Ovariectomy was performed 2 weeks after the 3rd administration of DMBA and 17 β -estradiol or progesterone was given 3 times a week, starting from 2 weeks after the 3rd administration of DMBA. Each value represents mean±S.D..

* p <0.05; ** p <0.01: significantly different from Group I

Table 4. Absolute and Relative Organ Weights in Inbred SD Female Rats Receiving Hormonal Treatment and A Multiple Administration of DMBA

Group	Absolute Organ weights			Relative organ weights		
	Pituitary (mg)	Adrenals (mg)	Ovaries (mg)	Pituitary (mg/g)	Adrenals (mg/g)	Ovaries (mg/g)
I Intact	94.3±21.62	342.9±63.41	613.1±170.87	0.51±0.101	1.89±0.527	3.27±0.702
II Ovariectomy (Intact)	111.2±12.50*	269.9±34.98**		0.44±0.063	1.08±0.209**	
III-1 17 β -estradiol (0.01 mg/body)	178.0±19.31**	298.0±19.65*	585.3±85.02	0.80±0.070**	1.40±0.153	2.74±0.406
III-2 17 β -estradiol (0.1 mg/body)	330.4±42.44**	230.0±37.89**	484.2±109.51	1.75±0.227**	1.22±0.174*	2.58±0.699
III-3 17 β -estradiol (1 mg/body)	356.8±159.67**	268.3±46.77**	342.2±125.49**	1.98±0.803**	1.51±0.240	1.94±0.739**
IV Progesterone (4 mg/body)	137.8±84.00	329.1±65.27	561.6±167.74	0.72±0.468	1.69±0.411	2.90±1.009

Ten mg DMBA were orally administered to 138 female rats 3 times at ages 28, 42 and 56 days. Ovariectomy was performed 2 weeks after the 3rd administration of DMBA and 17 β -estradiol or progesterone was given 3 times a week, starting from 2 weeks after the 3rd administration of DMBA.

Each value represents mean±S.D..

* p <0.05; ** p <0.01: significantly different from Group I

The weights of organs in Group B are shown in Table 4.

Absolute weights of the pituitary in Groups II, III-1, III-2 and III-3 and relative weights of the pituitary in Groups III-1, III-2 and III-3 increased significantly, compared with those in Group I. Absolute weights of the adrenal glands in

Groups II, III-1, III-2 and III-3 and relative weights of the adrenal glands in Groups II and III-2 decreased significantly, compared with those in Group I. Absolute and relative weights of the ovaries in Group III-3 decreased significantly, compared with those in Group I.

Fig. 1. Ten mg DMBA were orally administered to female rat once at age 42 days. Progesterone (4 mg) was given 3 times a week, starting from 4 weeks after administration of DMBA. Adenocarcinoma-Papillary type ($\times 70$)

Fig. 2. Ten mg DMBA were orally administered to female rat 3 times at ages 28, 42 and 56 days. Ovariectomy was performed 2 weeks after the 3rd administration of DMBA. Adenocarcinoma-Solid type ($\times 110$)

Table 5. Predominant Features of Mammary Carcinomas in Inbred SD Female Rats Receiving Hormonal Treatment and A Multiple Administration of DMBA (Group B)

Group	No. of tumors examined	Papillary type	Solid type	Secretory type
I Intact	158	73 (46.2%)	75 (47.5%)	10 (6.3%)
II Ovariectomy (Intact)	61	11 (18.0%)**	43 (70.5%)**	7 (11.5%)
III-1 17 β -estradiol (0.01 mg/body)	52	25 (48.1%)	24 (46.2%)	3 (5.8%)
III-2 17 β -estradiol (0.1 mg/body)	48	15 (31.3%)	20 (41.7%)	13 (27.1%)**
III-3 17 β -estradiol (1 mg/body)	68	15 (22.1%)**	26 (38.2%)	27 (39.7%)**
IV Progesterone (4 mg/body)	121	54 (44.6%)	63 (52.1%)	4 (3.3%)

Ten mg DMBA were orally administered to 138 female rats 3 times at ages 28, 42 and 56 days. Ovariectomy was performed 2 weeks after the 3rd administration of DMBA and 17 β -estradiol or progesterone was given 3 times a week, starting from 2 weeks after the 3rd administration of DMBA. Each value represents mean $\pm$ S.D..

* $p < 0.05$; ** $p < 0.01$: significantly different from Group I

Histopathology and immunohistology

Mammary carcinomas in Group B were subclassified into 3 types, papillary type (Fig. 1), solid type (Fig. 2) and secretory type (Fig. 3) by predominant histologic features. Predominant features of mammary carcinomas in Group B were shown in **Table 5**. Rates of mammary carcinomas of solid type and secretory type increased in Group II and Groups III-2 and 3, respectively, compared with that of Group I. The lumina in the mammary glands in Groups II-3, II-4, III-2 and III-3 of A and III-2 and III-3 of B contained milky secretions. Immunohistochemical examination of pituitaries of Group B revealed that prolactin-positive cells increased in Groups III-2 and III-3 (Fig. 4), compared with that of Group I (Fig. 5).

Transplantation

The growth of mammary carcinomas transplanted into ovariectomized rats with injections of 0.01(R-2) or 1.0 mg E₂ (R-3) or with no injection (R-1) is shown in **Table 6**. According to tumorigenesis in rats with those hormonal treatments, mammary carcinomas were classified into one of the four categories described below.

Type A tumor: tumors developed greatly in R-2 but slightly or not in R-1 and R-3.

Type B tumor: tumors developed greatly in R-1 rats but slightly or not in R-2 and R-3.

Type C tumor: tumors developed greatly in R-3 rats but slightly or not in R-2 and R-1.

Type D tumor: tumors developed greatly in R-1, R-2 and R-3.

Fig. 3. Ten mg DMBA were orally administered to female rat 3 times at ages 28, 42 and 56 days. 17 β -estradiol (1 mg) was given 3 times a week, starting from 2 weeks after the 3rd administration of DMBA. Adenocarcinoma-Secretory type ($\times 110$)

Fig. 4. Expression of prolactin in cytoplasm of many cells in a pituitary of the rat with injections of high doses of E. Immunohistochemical method. ($\times 690$)

Fig. 5. Expression of prolactin in a few cells of the pituitary in a intact rat. Immunohistochemical method. ($\times 690$)

Table 6. Growth of Mammary Carcinomas Transplanted into Rats under Various Hormonal Conditions

Group	No. of tumors examined	A-Type			B-Type			C-Type			D-Type		
		Recipient			Recipient			Recipient			Recipient		
		R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3
		+/-	++	+/-	++	+/-	+/-	+/-	+/-	++	++	++	++
I	Intact	37	16 (43.2%)		5 (13.5%)			9 (24.3%)			7 (18.9%)		
II	Ovariectomy	19	5 (26.3%)		2 (10.5%)			4 (21.1%)			8 (42.1%)		
III-1	17 β -estradiol (0.01 mg/body)	20	8 (40.0%)		1 (5.0%)			9 (45.0%)			2 (10.0%)		
III-2	17 β -estradiol (0.1 mg/body)	13	5 (38.5%)		2 (15.4%)			4 (30.8%)			2 (15.4%)		
III-3	17 β -estradiol	21	6 (28.6%)		0 (0.0%)			15** (71.4%)			0* (0.0%)		
IV	Progesterone	9	4 (44.4%)		3 (33.3%)			1 (11.1%)			1 (11.1%)		

DMBA (3 administration) -induced mammary carcinomas were transplanted into ovariectomized rats, which were then given hormonal treatment (R-1: Intact, R-2: 17 β -estradiol, 0.01 mg, i.m., 3 times/week, R-3: 17 β -estradiol, 1 mg, i.m., 3 times/week).

++ : Great tumorigenesis, +/- : Slight tumorigenesis or nontumorigenesis.

* $p < 0.05$; ** $p < 0.01$: significantly different from Group I

The rate of Type C tumors increased significantly in Group III-3, compared with that in Group I.

Discussion

The results of the present study (Table 1) show that a single administration of 10 mg DMBA induces a few mammary carcinomas in intact female rats and rats with injections of suitable doses of E_2 or P. Mammary carcinogenesis was completely suppressed in ovariectomized rats and in rats with injections of high doses of E_2 . These findings suggest that a single administration of a low dose of DMBA induces only a few carcinoma cells and that the progression of almost all mammary carcinoma cells is estrogen-dependent because the progression of mammary carcinomas is completely under the influence of the dosage of estrogen.

Estrogen directly affects dopamine neurons in the hypothalamus and thus inhibits the production of prolactin release inhibiting factor, leading to the secondary secretion of prolactin from the pituitary; and ovariectomy reduces the secretion of prolactin^{7,8} while progesterone partially inhibits the effect of estrogen on prolactin secretion⁷. In the present experiment, pituitary weights increased greatly in rats injected with E_2 and decreased in ovariectomized rats. Histopathological and immunohistochemical examination also revealed that prolactin-positive cell increased in pituitary glands of rats with injections of high doses of E_2 and lactation in the mammary glands of rats that were injected with high doses of E_2 but not in ovariectomized rats or those injected with P. It was, therefore, estimated that serum prolactin levels increased in rats injected with E_2 and decreased in ovariectomized rats or those injected with P.

It has been reported that mammary carcinogenesis of DMBA is strongly influenced not only by estrogen and progesterone, which are secreted from the ovaries, but also by prolactin secreted from the pituitary^{1,2}. In neonatally androgenized rats, corpora lutea were absent⁹. It has been

shown in our previous studies that injections of P induced a marked increase in the incidence of mammary carcinomas from that in the androgenized controls (3.4%) in the presence of ovaries (90%) but not in the absence of ovaries (8.7%)¹⁰. P, therefore, stimulates the growth of some DMBA induced mammary carcinomas and the stimulatory effect depends on the action of E_2 . Furth and other researchers have clarified that prolactin plays an extremely important role in mammary carcinogenesis in mice and rats^{11,12}. The growth of established carcinogen-induced rat mammary carcinomas also appears to be regulated by steroid and pituitary hormonal interactions. The ovariectomy of rats bearing carcinogen induced mammary carcinomas resulted in a prompt and prolonged regression of these tumors¹³⁻¹⁵. Concurrent enhancement of prolactin secretion by the placement of median-eminence hypothalamic lesions in these animals prevents this regression¹⁶. This enhanced mammary tumor growth, however, would not persist for long periods unless ovaries were re-implanted into these animals¹⁷. Accordingly, the daily injection of ovine prolactin or human placental lactogen into ovariectomized-adrenalectomized or ovariectomized-adrenalectomized-hypophysectomized rats bearing carcinogen-induced mammary tumors also resulted, initially at least, in, the increased growth of these tumors¹⁸⁻²⁰. These results suggest that prolactin can stimulate growth of carcinogen-induced rat mammary tumors in animals lacking ovarian steroids, but for persistent growth of these tumors, despite hyperprolactinemia, ovarian steroids may be essential. An exception to this concept is the study by Kim and Furth²¹ showing that hormonal secretions from a transplantable pituitary tumor are capable of promoting growth of carcinogen (MCA)-induced mammary tumors in ovariectomized Fisher and Wistar rats for periods of several months. It is probable that the growth dependency of carcinogen-induced rat mammary tumors on prolactin and ovarian hormones will vary from tumor to tumor, i.e., some tumors will require substantially greater or lesser amounts of prolactin and/or

ovarian hormones for an optimal growth process^{22,23}. Hypophysectomy caused a rapid regression of the mammary tumors. The administration of estrogens to the tumor-bearing, hypophysectomized rats did not cause a resumption in tumor growth^{20,24}. Most hormone-responsive mammary tumors in rodents appear to require participation of both the pituitaries and ovaries for optimal growth processes. In the present experiment (Group A), the development of mammary carcinomas in ovariectomized rats injected with 0.01 mg E₂ and in rats injected with 0.01 mg E₂ and /or P were equivalent to that in the intact control group. These findings suggest that treatment with suitable doses of E₂ may trigger mutual interaction of effects of estrogen and prolactin and/or progesterone, thus leading to mammary carcinogenesis. The mechanism by which high doses of estrogen inhibit mammary carcinogenesis despite high levels of prolactin (Groups II-3, III-2 and III-3) is not known but there are a few reports of studies on the mechanism. High doses of estrogen reduce the prolactin receptors in mammary carcinoma cells, suggesting the mechanism^{25,26}. But, since high doses of estrogens suppressed DNA synthesis of organ cultures of rodent mammary tumors, whether prolactin was added to the culture media or not, other mechanisms of steroid treatment appear to be operating as well^{27,28}.

The results of the present study (Table 3) show that a multiple administration of DMBA induces high incidences of mammary carcinomas in intact female rats but that the number of mammary carcinomas per rat decreases in ovariectomized rats injected with high doses of E₂. It has been reported in our previous studies that the number of apoptotic cells and Apop-Tag positive cells of mammary carcinomas induced by the multiple administration of DMBA is greater in rats treated with high doses of E₂ than in intact rats²⁹. According to the findings of our previous studies²⁹⁻³¹, inhibitory effects of high doses of estrogen on the growth of mammary carcinomas seem to be due to accelerated apoptosis of carcinoma cells.

Those findings (Table 6), obtained from examinations of the tumorigenesis of transplanted tumor cells in various hormonal conditions, showing that the rate of Type C tumor which could grow in rats with injections of high doses estrogen increased in Group III-3 suggest that the hormonal status of the development process may be one of factors which affects the characteristics of hormone dependency and that the multiple administration of DMBA may induce large numbers of carcinoma cells with varying dependency on hormones (like in Group I of B, Table 6) and carcinoma cells may be selected in hormonal conditions during progression.

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References

- Huggins C, Grand LC, and Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* 1961; **189**: 204-207.
- Meites J. Relation of prolactin and estrogen to mammary tumorigenesis in the rat. *J Natl Cancer Inst* 1972; **48**: 1217-1224.
- Dao TL. The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. *Cancer Res* 1962; **22**: 973-981.
- Sylvester PW, Aylsworth CF, and Meites J. Relationship of hormones to inhibition of mammary tumor development by underfeeding during the "critical period" after carcinogen administration. *Cancer Res* 1981; **41**: 1384-1388.
- Sylvester PW, Aylsworth CF, Van Vugt DA, and Meites J. Influence of underfeeding during the "critical period" or thereafter on carcinogen-induced mammary tumors in rats. *Cancer Res* 1982; **42**: 4943-4947.
- Mainland D and Murray IM. Tables for use in fourfold contingency tests. *Science* 1952; **116**: 591-594.
- Chen CL and Meites J. Effects of estrogen and progesterone on serum and pituitary prolactin levels in ovariectomized rats. *Endocrinology* 1970; **86**: 503-505.
- Meites J and Nicoll CS. Adenohypophysis: Prolactin. *Ann Rev Physiol* 1966; **28**: 57-88.
- Yoshida H, Kadota A, Fukunushi R, and Matsumoto K. Induction of mammary dysplasia and mammary carcinoma in neonatally androgenized female rats by 7, 12-dimethylbenz[a]anthracene. *J Natl Cancer Inst* 1980; **64**: 1105-1112.
- Yoshida H, Fukunushi R, Kato Y, and Matsumoto K. Progesterone-stimulated growth of mammary carcinomas induced by 7, 12-dimethylbenz[a]anthracene in neonatally androgenized rats. *J Natl Cancer Inst* 1980; **65**: 823-828.
- Furth J. The role of prolactin in mammary carcinogenesis. In: Human Prolactin, Pasteels JL and Robin C (eds.), New York: Excerpta Medica/American Elsevier Publishing Co. Inc., 223-248, 1973.
- Welsch CW and Nagasawa H. Prolactin and murine mammary tumorigenesis: a review. *Cancer Res* 1977; **37**: 951-963.
- Dao TL and Sunderland H. Mammary carcinogenesis by 3-methylcholanthrene I. Hormonal aspects in tumor induction and growth. *J Natl Cancer Inst* 1959; **23**: 567-585.
- Huggins C, Briziarelli G, and Sutton H. Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors. *J Exptl Med* 1959; **109**: 25-42.
- Welsch CW. Growth inhibition of rat mammary carcinoma induced by cis-platinum diammino-dichloride-II. *J Natl Cancer Inst* 1971; **47**: 1071-1078.
- Welsch CW, Clemens JA, and Meites J. Effects of hypothalamic and amygdaloid lesions on development and growth of carcinogen-induced mammary tumors in the female rat. *Cancer Res* 1969; **29**: 1541-1549.
- Sinha D, Cooper D, and Dao TL. The nature of estrogen and prolactin effect on mammary tumorigenesis. *Cancer Res* 1973; **33**: 411-414.
- Nagasawa H and Yanai R. Effects of prolactin or growth hormone on growth of carcinogen-induced mammary tumors of Adreno-ovariectomized rats. *Intern J Cancer* 1970; **6**: 488-495.
- Nagasawa H and Yanai R. Effect of human placental lactogen on growth of carcinogen-induced mammary tumors in rats. *Intern J Cancer* 1973; **11**: 131-137.
- Pearson OH, Llerena O, Llerena L, Molina A, and Butler T. Prolactin-dependent rat mammary cancer: a model for man? *Trans Assoc Am Physicians* 1969; **82**: 225-238.
- Kim U and Furth J. Relation of mammary tumors to mammotropes. II. Hormone responsiveness of 3-methylcholanthrene induced mammary carcinomas. *Proc Soc Exptl Biol Med* 1960; **103**: 643-645.
- Bradley CJ, Kledzik GS, and Meites J. Prolactin and estrogen dependency of rat mammary cancers at early and late stages of development. *Cancer Res* 1976; **36**: 319-324.
- Nagasawa H, Chen CL, and Meites J. Relation between growth of carcinogen-induced mammary cancers and serum prolactin values in rats. *Proc Soc Exptl Biol Med* 1973; **142**: 625-627.
- Sterental A, Dominguez JM, Weissman C, and Pearson OH.

- Pituitary role in the estrogen dependency of experimental mammary cancer. *Cancer Res* 1963; **23**: 481-484.
25. Kledzik GS, Bradley CJ, Marshall S, Campbell GA, and Meites J. Effects of high doses of estrogen on prolactin-binding activity and growth of carcinogen-induced mammary cancers in rats. *Cancer Res* 1976; **36**: 3265-3268.
26. Shigaki N, Kimura M, Takano S, Fujino N, and Akagi M. Reduction of estrogen and prolactin receptors in 7, 12-Dimethylbenz[a]anthracene-induced rat mammary tumor by high doses of estrogen. *Japanese Journal of Surgery* 1987; **17**: 395-401.
27. Turkington RW and Hilf R. Hormonal dependence of DNA synthesis in mammary carcinoma cells *in vitro*. *Science* 1968; **160**: 1457-1459.
28. Welsch CW and Rivera EM. Differential effects of estrogen and prolactin on DNA synthesis in organ cultures of DMBA-induced rat mammary carcinoma. *Proc Soc Exptl Biol Med* 1972; **139**: 623-626.
29. Okasaki K, Omachi K, Sato K, Funato M, Nagata R, and Yoshida H. Growth inhibition of a high dose of 17β -estradiol and ovariectomy on 7, 12-Dimethylbenz(a)anthracene-induced mammary carcinomas in female rats. *J Toxicol Pathol* 1998; **11**: 257-262.
30. Yoshida H, Fukuhara J, Yamashi S, Yoshida A, Fukunishi R, Tsuji M, and Matsumoto K. Effects of pharmacological doses of estrogen on ovary-independent rat mammary carcinoma containing estrogen and progesterone receptors. *Cancer Res* 1983; **43**: 2311-2316.
31. Yoshida H, Yoshida A, Yamada M, Yamanaka K, Kitamura Y, and Matsumoto K. Regression with secretory changes of ovary-independent rat mammary carcinoma by pharmacological doses of estrogen. *Jpn J Cancer Res (Gann)* 1985; **76**: 771-778.