Growth Inhibition of a High Dose of 17 β -Estradiol and Ovariectomy on 7, 12-Dimethylbenz(a)anthracene-induced Mammary Carcinomas in Female Rats

Keikou Okasaki¹, Katsumi Omachi¹, Kenichi Sato¹, Mamoru Funato¹,
Ryoichi Nagata², and Hiroki Yoshida¹

¹The Department of Pathology I, Faculty of Medicine, Kagoshima University 8-35-1 Sakuragaoka, Kagoshima 890-8520 Japan, ²Shin Nippon Biomedical Laboratories, Ltd. 2438 Miyanoura, Yoshida, Kagoshima 891-1394 Japan

Abstract: In this study the inhibitory effect of a high dose of estrogen on mammary carcinoma growth was investigated, especially in relation to apoptosis and mitosis, and compared this to the effects of ovariectomy. A transplantable mammary carcinoma with estrogen receptors was induced in a female Sprague-Dawley rat by multiple intragastric intubations of 7, 12-dimethylbenz(a)anthracene. The mammary carcinoma was then transplanted into female rats, and on the 21st day after the transplantation the rats were divided into three groups. Rats in two of these groups were injected with either vehicle or 1.0 mg of 17 β -estradiol three times a week, and rats in the remaining group were ovariectomized. Rats were sacrificed on either the 1st, 7th, 14th, or 21st day after the initial administration or ovariectomy. The growth of transplanted mammary carcinomas was suppressed in ovariectomized rats and rats treated with a high dose of 17 β -estradiol. In the 17 β -estradiol treated rats, the incidences of apoptotic cells and Apop Tag positive cells were higher than those of the intact control on the 7th to 21st day after the initial administration, and the incidences of mitotic cells and 5-Bromodeoxyuridine staining positive cells were higher only on the 7th day after the initial administration. Conversely, there was no difference between incidences of apoptotic cells and Apop Tag positive cells between the ovariectomized rats and the intact control throughout the treatment period, and incidences of mitotic cells and 5-Bromodeoxyuridine staining positive cells were lower in the ovariectomized rats on the 7th to 21st day after the ovariectomy. These results indicate that the growth inhibition of the mammary carcinoma in rats treated with a high dose of 17 β -estradiol was related to apoptosis. In contrast, a decrease of cell proliferation is considered to be the mechanism responsible for the inhibitory effect of ovariectomy on the growth of the mammary carcinoma. (J Toxicol Pathol 1998; 11: 257~262)

Key words: mammary carcinoma, 7, 12-dimethylbenz(a)anthracene, rat, 17 β -estradiol, apoptosis

Introduction

It has been generally accepted that endocrine treatments such as estrogen removal and the addition of a high dose of estrogen are effective in patients with estrogen receptor-positive breast cancer (1, 2). Additionally, it is well known that a high dose of estrogen causes regression of rat mammary carcinomas, which were induced by administration of 7, 12 dimethylbenz(a)anthracene (DMBA) (3, 4, 5, 6, 7, 8, 9). However, there are only few reports which focus on the mechanism of effect of such hormonal treatment (9). This study, therefore, investigated the inhibitory effect of a high dose of estrogen on carcinoma growth, especially in relation to apoptosis or mitosis, and compared it to the effects of ovariectomy.

Materials and Method

Animals and maintenance

Inbred Sprague-Dawley (SD) female rats, maintained at the Faculty of Medicine, Kagoshima University, were used in the experiments. The animals were given a commercial diet (CE-2, CLEA Japan, Inc.) and tap water *ad libitum*. The room temperature was maintained at 23 $\pm$ 2°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 carcinoma, transplantation, and hormonal treatment

Ten milligrams of DMBA (Wako Pure Chemical Industries, Ltd., Japan) were orally administered to an inbred SD female rat 3 times, at 28, 42, and 56 days of age. Five weeks after the third administration of DMBA, a DMBA induced mammary carcinoma was removed from the rat and minced. Approximately 50 to 80 mg of the minced carcinoma tissue were transplanted at the upper and middle milk lines of intact inbred SD female rats of 70 to 80 days old via subcutaneous injection (No. 13 trocar). On the 21st day after the transplantation, 7 to 8 rats were allocated to three groups, two groups were injected intramuscularly with either 0.1 ml of

岡崎啓幸, 大町勝己, 里 健一, 船戸 護, 永田良一, 吉田浩己

Received: 11 September 1998, Accepted: 5 November 1998

Mailing address:

This work was supported in parts by the Kodama Fund for Medical Research.

Correspondence to Hiroki Yoshida; The Department of Pathology I, Faculty of Medicine, Kagoshima University 8-35-1, Sakuragaoka, Kagoshima 890-8520 Japan.

Fax: 099-264-6348, Phone: 099-275-5260

Fig. 1. Estrogen receptors were expressed in nuclei of 7, 12-Dimethylbenz(a)anthracene-induced mammary carcinoma cells. Immunohistochemical method. $\times 128$

Table 1. Growth of the mammary carcinomas in rats with the E_2 treatment (3 times/week) or the ovariectomy

Day/ Treatment	None (Vehicle)	E_2 treatment (1 mg/body)	Ovariectomy
1	0.995 $\pm$ 0.701 (21)	1.029 $\pm$ 0.809 (20)	1.315 $\pm$ 0.506 (13)
7	2.033 $\pm$ 1.591 (21)	1.842 $\pm$ 1.245 (20)	1.654 $\pm$ 0.610 (13)
14	3.043 $\pm$ 1.991 (21)	2.205 $\pm$ 1.028 (20)	1.500 $\pm$ 0.502* (13)
21	4.264 $\pm$ 2.975 (21)	2.380 $\pm$ 1.027* (20)	0.932 $\pm$ 0.341** (13)

Figures indicate mean $\pm$ S.D. of the carcinoma size (cm², multiplied major and minor axes) and those in the parenthesis are no. of samples.

Day; Days after the start of dosing or the ovariectomy.

*: $p < 0.05$, **: $p < 0.01$, significantly different from the control by unpaired t -test (two tailed).

vehicle (combination of saline, 0.4% Tween 80, 0.5% carboxymethylcellulose, and 0.9% benzyl alcohol) or 1.0 mg of 17 β -estradiol (E_2 , Sigma Chemical Co., USA) dissolved in 0.1 ml of the vehicle, three times a week. Rats in the remaining group were ovariectomized. The rats were sacrificed on either the 1st day (Day 1), the 7th day (Day 7), the 14th day (Day 14) or the 21st day (Day 21) after the first injection of E_2 or the ovariectomy, and the carcinomas were removed for examination.

Immunohistochemical staining for estrogen receptors

Parts of the mammary carcinomas used for transplantation were fixed in 10% phosphate-buffered formalin (PBF) for up to 24 hours at room temperature and embedded in paraffin. Sections were cut from paraffin embedded tissue blocks, deparaffinized and rehydrated. They were placed in a beaker filled with 500 ml of phosphate-buffered saline (PBS), pH 7.2, heated in a microwave oven at 500 W for 5 min. twice, and then subjected to the standard avidin-biotin complex immunoperoxidase assays using the Vectastain ABC kit (Vector Laboratories, USA). Deparaffinized and rehy-

drated sections were treated with 0.3% hydrogen peroxide in methanol solution to quench endogenous peroxidase activity, and were subsequently incubated at room temperature with the following reagents (with PBS washes in between): 1.0% normal bovine serum albumin (BSA) for 30 min., primary antiserum diluted in PBS with 1.0% BSA (Immunotech, France) for 30 min., biotinylated goat anti-mouse antiserum for 30 min., ABC complex for 30 min., and 3, 3'-diaminobenzidine tetrachloride (DAB) containing 0.05% hydrogen peroxide for 5 min. The sections were then washed, counterstained, dehydrated, cleared in xylene, and mounted. Controls were set up with PBS containing 1.0% BSA instead of the primary antisera.

Measurement of size and weight of the transplanted tumors

The transplanted carcinomas in rats of each group were measured on their major and minor axes by palpation on Days 1, 7, 14, and 21, and the size was calculated from the multiplied major and minor axes. At sacrifice on Day 28, the carcinomas in each group were removed and weighed.

Apoptotic, mitotic, Apop Tag-positive and Br-dU-positive cell counts

a. Hematoxylin and eosin (H.E.) staining

After removal, the transplanted carcinomas were fixed in 10% PBF, dehydrated, embedded in paraffin wax, sectioned to 5 μ m, and stained with H.E. stain for light microscopy.

b. Apop TagTM staining

Apoptosis was detected using the Apop TagTM In Situ Apoptosis Detection Kit (Oncor, Inc., USA) (11, 12, 13, 14, 15). Mammary carcinomas were fixed in NBF for up to 24 hours at 4°C and embedded in paraffin. The paraffin section was deparaffinized, specimens were rehydrated, and Proteinase K (20 μ g/ml) was applied directly to slide mounted specimens for 15 min. at room temperature and washed. After washing, two drops of equilibration buffer were applied directly to the slide specimen in a humidity chamber for 5 to 10 min. at room temperature. Working strength TdT Enzyme (mixture of TdT Enzyme and reaction buffer) was then immediately pipetted onto the section, and the slide was again incubated in a humidity chamber at 37°C for 1 hour. After the incubation, the slide was put in a coplin jar containing pre-warmed working strength stop/wash buffer, incubated again for a further 30 min. at 37°C, and washed in PBS. Anti-Digoxigenin-Peroxidase was then applied to the slide for 30 min. at room temperature and washed in PBS at room temperature. Enough newly prepared 0.05% DAB substrate solution to completely cover the specimen was then immediately applied, and the specimen was stained for 3 to 6 min. at room temperature. The specimen was washed in distilled water, counterstained in methyl green for 10 min. at room temperature, washed in distilled water again, dehydrated, and mounted. A normal rodent small intestine was used as the positive control.

c. 5-Bromodeoxyuridine (Br-dU) staining

In order to clarify the cell proliferation features, the 6 to 8 transplanted carcinomas were excised from 3 or 4 rats in each group, 1 hour after Br-dU (20 mg/kg body weight) was administered intraperitoneally. The removed carcinomas were placed in 70% ethanol for 2 hours and then routinely

processed for immunohistologic examination with monoclonal anti-Br-dU antibody. Avidin-biotin complex immunoperoxidase assays were performed with the Vectastain ABC kit (10). Deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol solution to quench endogenous peroxidase antibodies, and subsequently incubated at room temperature using the following reagents, interspersed with PBS washes: 1% BSA for 30 min., anti-Br-dU diluted in PBS with 1.0% BSA (1:50) for 30 min., biotinylated goat anti-mouse antiserum for 30 min., ABC complex for 30 min., and DAB containing 0.05% hydrogen peroxide for 5 min. The sections were then washed, counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted. Comparative controls were set up with PBS containing 1.0% BSA instead of the primary antisera. Monoclonal anti-Br-dU (Becton Dickinson Immunocytotechnology System, USA) was derived from hybridization of mouse Sp2/0-Ag14 cells with spleen cells of BALB/c mice immunized with iodouridine-conjugated ovalbumin.

d. Cell counts

After preparation of H.E., Br-dU, and Apop Tag stained specimens, the number of apoptotic cells, mitotic cells, Apop Tag positive cells, and Br-dU positive cells was counted in five distinct areas per carcinoma using intermediate or high magnification light microscopy. Each area of each specimen contained approximately three hundred to eight hundred carcinoma cells. The incidences (%) of apoptotic cells, mitotic cells, Apop Tag positive cells, or Br-dU positive cells in each group were analyzed statistically using an unpaired Student's *t*-test (two tailed).

Ultrastructure

Glutaraldehyde-fixed and osmium tetroxide post-fixed samples, embedded in epoxy resin, were cut to 60 nm, stained with uranyl acetate and lead nitrate, and examined in order to confirm the apoptosis on the H.E. stained preparation with the Jeol JEM 1200 EX transmission electron microscope (Japan).

Results

Estrogen receptor staining

The immunohistochemical examination revealed positive staining of estrogen receptors in almost all nuclei of DMBA-induced mammary carcinoma cells (Fig. 1), indicating that the carcinoma had estrogen receptors.

Inhibitory effects of a high dose of E_2 treatment or ovariectomy on the growth of transplanted DMBA-induced mammary carcinomas

The size of mammary carcinomas which were transplanted in rats that were then treated with E_2 or ovariectomized, are shown in Table 1. The growth of the transplanted carcinomas in the E_2 treated group was suppressed significantly ($p < 0.05$) on Day 21 after the first treatment, compared with that of the intact control. In the ovariectomized rats, the growth was also suppressed significantly ($p < 0.05$ or 0.01) on Days 14 and 21 after ovariectomy. On Day 21, the sizes of the carcinomas in the E_2 treated and ovariectomized rats were 55.8% and 21.9% of the intact control, respectively. At sacrifice the weights of mammary carcinomas in the intact control rats, in rats with E_2 treatment,

and rats with ovariectomy were 2.854 ± 2.600 (21 samples), 1.473 ± 0.790 (20), and 0.304 ± 0.141 g (13), respectively, and the weights in the E_2 treated and ovariectomized rats were significantly lower than in the intact control rats ($p < 0.05$, or 0.01).

Incidence of apoptotic cells and Apop Tag positive cells

The incidence of apoptotic cells in the H.E. stained preparations and Apop Tag positive cells is shown in Tables 2 and 3. Apoptotic cells were characterized microscopically by cell shrinkage, condensed and fragmented nuclei, and eosinophilic apoptotic bodies, and electron-microscopically by condensation of chromatin and fragmentation of nucleus (Fig. 2 and 3). The incidences of apoptotic cells in the E_2 treated rats were significantly higher ($p < 0.01$) than those of the intact control rats on Days 7, 14, and 21 after the first treatment. There was no difference between the incidences in the ovariectomized rats and the intact control rats throughout the treatment period. Apop Tag positive cells in the E_2 treated rats increased significantly ($p < 0.05$) on Days 7, 14,

Table 2. Incidence of apoptotic cells (% of carcinoma cells) in the mammary carcinomas in rats with the E_2 treatment (3 times/week) or the ovariectomy

Day/ Treatment	None (Vehicle)	E_2 treatment (1 mg/body)	Ovariectomy
1	0.65 ± 0.25 (10)	0.83 ± 0.34 (10)	0.68 ± 0.26 (10)
7	0.60 ± 0.07 (10)	$1.19 \pm 0.42^{**}$ (10)	0.81 ± 0.40 (10)
14	0.58 ± 0.32 (10)	$1.43 \pm 0.38^{**}$ (10)	0.75 ± 0.27 (10)
21	0.74 ± 0.37 (10)	$1.31 \pm 0.38^{**}$ (10)	0.64 ± 0.42 (10)

Figures indicate mean $\pm$ S.D. and those in the parenthesis are no. of samples.

Day; Days after the start of dosing or the ovariectomy.

$^{**}p < 0.01$, significantly different from the control by unpaired *t*-test (two tailed).

Table 3. Incidence of Apop-Tag positive cells (% of carcinoma cells) in the mammary carcinomas in rats with the E_2 treatment (3 times/week) or with the ovariectomy

Day/ Treatment	None (Vehicle)	E_2 treatment (1 mg/body)	Ovariectomy
1	0.87 ± 0.26 (12)	0.70 ± 0.29 (12)	0.95 ± 0.47 (12)
7	0.53 ± 0.27 (16)	$1.17 \pm 0.22^*$ (14)	0.73 ± 0.30 (15)
14	0.64 ± 0.34 (26)	$1.16 \pm 0.32^*$ (12)	0.69 ± 0.24 (16)
21	0.76 ± 0.31 (10)	$1.59 \pm 0.43^*$ (14)	1.04 ± 0.31 (6)

Figures indicate mean $\pm$ S.D. and those in the parenthesis are no. of samples.

Day; Days after the start of dosing or the ovariectomy.

$^*p < 0.05$, significantly different from the control by unpaired *t*-test (two tailed).

Fig. 2. Apoptotic carcinoma cells with condensed and fragmented nuclei (arrow head) and apoptotic bodies (small arrow), and a carcinoma cell with a mitotic figure (large arrow) in a rat treated with high doses of E_2 . H.E. Stain. $\times 256$

Fig. 3. Ultrastructural characteristics of an apoptotic cell in the mammary carcinoma in a rat treated with high doses of E_2 . The apoptotic cell is characterized by condensation of chromatin and fragmentation of nucleus. Bar=2 μm

and 21 after the first treatment compared to those of the intact control rats (Fig. 4). There was no difference between the incidences in the ovariectomized rats and the intact control rats throughout the treatment period.

Incidence of mitotic cells and Br-dU positive cells

The incidence of mitotic cells in the H.E. stained preparation and Br-dU positive cells is shown in **Tables 4** and **5**. The incidence of mitotic cells (Fig. 2) in the E_2 treated rats on Day 7 after the first treatment was significantly higher ($p < 0.01$) than that of the intact control rats. In the ovariectomized rats, incidences of the mitotic cells were significantly lower ($p < 0.05$ or 0.01 , excluding Day 7) than those of the intact control from Day 7 to Day 21 after ovariectomy.

The incidence of Br-dU positive cells in the E_2 treated rats was significantly higher than that of the intact control rats on Day 7 ($p < 0.01$) after the first treatment (Fig. 5). In the ovariectomized rats, Br-dU positive cell incidence decreased

Fig. 4. Several Apop Tag positive cells (arrow) in the mammary carcinoma in a rat treated with high doses of E_2 . Apop Tag stain $\times 256$

Table 4. Incidence of mitotic cells (% of carcinoma cells) in the mammary carcinomas in rats with the E_2 treatment (3 times/week) or the ovariectomy

Day/ Treatment	None (Vehicle)	E_2 treatment (1 mg/body)	Ovariectomy
1	0.87 ± 0.27 (10)	0.98 ± 0.22 (10)	0.84 ± 0.26 (10)
7	0.51 ± 0.14 (10)	$0.88 \pm 0.29^{**}$ (10)	0.38 ± 0.09 (10)
14	0.73 ± 0.22 (10)	0.80 ± 0.36 (10)	$0.50 \pm 0.16^*$ (10)
21	0.92 ± 0.38 (10)	1.02 ± 0.47 (10)	$0.29 \pm 0.29^{**}$ (10)

Figures indicate mean $\pm$ S.D. and those in the parenthesis are no. of samples.

Day; Days after the start of dosing or the ovariectomy.

*: $p < 0.05$, **: $p < 0.01$, significantly different from the control by unpaired t -test (two tailed).

Table 5. Incidence of Br-dU positive cells (% of carcinoma cells) in the mammary carcinomas in rats during the E_2 treatment (3 times/week) or the ovariectomy

Day/ Treatment	None (Vehicle)	E_2 treatment (1 mg/body)	Ovariectomy
1	10.96 ± 1.12 (8)	10.38 ± 2.78 (12)	10.66 ± 2.49 (12)
7	12.95 ± 4.44 (12)	$17.27 \pm 3.04^{**}$ (15)	$9.08 \pm 2.29^{**}$ (16)
14	11.31 ± 2.81 (8)	14.15 ± 4.78 (8)	9.70 ± 3.32 (12)
21	18.01 ± 2.98 (6)	16.96 ± 3.21 (7)	$9.18 \pm 4.78^{**}$ (6)

Figures indicate mean $\pm$ S.D. and those in the parenthesis are no. of samples.

Day; Days after the start of dosing or the ovariectomy.

** $p < 0.01$, significantly different from the control by unpaired t -test (two tailed).

Fig. 5. Br-dU positive cells in the mammary carcinoma in a rat treated with high doses of E_2 . Immunohistochemical method. $\times 256$

significantly ($p < 0.01$, excluding Day 14) from Day 7 to Day 21 after ovariectomy.

Discussion

The growth of mammary carcinomas with estrogen receptors, which have been induced in female rats given 20 mg DMBA once by gastric intubation at 50 days of age, is ovary-dependent because growth is inhibited by ovariectomy. The growth of tumors is suppressed in females given injections of high dose of E_2 (5, 8). Conversely, the growth of mammary carcinomas with estrogen receptors, which have been induced in male rats given 10 mg DMBA at 14-day intervals for 14 weeks starting from 28 days of age, is ovary-independent because growth is not suppressed by ovariectomy. However, the growth of mammary carcinomas induced in male rats is inhibited by injections of high dose of E_2 (8). We have also reported that ovary-independent mammary carcinomas are induced in gonadectomized males and females by multiple administration of DMBA (10). The present study showed that a mammary carcinoma, which was induced in a female rat given 10 mg DMBA at 14-day intervals for 4 weeks starting from 28 days of age, contained estrogen receptors and their growth was suppressed significantly by ovariectomy and injections of high dose of E_2 . Therefore, the growth of the mammary carcinoma used in the present study is ovary-dependent. According to the findings of our present and previous studies, it is suggested that the hormonal environment during the neoplastic process might exert a major influence on the biological characteristics of induced mammary carcinomas.

In order to clarify the mechanism of inhibitory effects of E_2 or ovariectomy on DMBA-induced mammary carcinomas, we examined apoptosis and cell proliferation of the mammary carcinomas in female rats with such treatment. Apoptosis, in the present study, was identified morphologically by light microscopy and transmission electron-microscopy (16, 17). DNA fragmentation was also confirmed by Apop Tag stain method. In order to detect the cell proliferation of carcinoma cells, carcinomas were examined immunohistochemically with anti-Br-dU antibody. The results of this morphological examination demonstrated that in female rats with a high dose of E_2 both

mitotic cells and Br-dU positive cells increased slightly on only Day 7, but both apoptotic cells and Apop Tag positive cells increased significantly during Days 7 to 21. The results indicated that the inhibitory effect of high dose of E_2 was related mainly to continuous enhancement of apoptosis, although the reason why both mitotic cells and Br-dU positive cells increased on only Day 7 is unknown and then further study is necessary.

Although E_2 is well known as a hormonal growth factor for mammary carcinomas (18), there are few reports about the apoptosis-induction effect of a high dose of E_2 treatment on mammary carcinoma cells (9). A transplantable rat mammary carcinoma with estrogen and progesterone receptors, MT6, obtained from a mammary carcinoma in a male rat was an ovary-independent and prolactin-independent tumor. However, the growth of MT6 was inhibited by a high dose of E_2 . Uptake values of 5- $[^{125}I]$ iodo-2'-deoxyuridine per g of living tumor, used as an index of cell proliferation, were not significantly reduced by treatment with a high dose of E_2 . When tumor cell proliferation was evaluated by labeling with 5- $[^{125}I]$ iodo-2'-deoxyuridine just before the treatment, the radioactivity incorporated by the whole tumor was decreased in the E_2 treated rats. Secretory changes characterized by secretion in the lumina, vacuolation in the cytoplasm, marked increase in vesicular endoplasmic reticulum, accumulation of mature protein secretory granules, and abundance of lipid droplets were observed in MT6 cells in those rats. These findings suggest that the inhibitory effect of a high dose of E_2 on the growth of MT6 tumors is mainly due to secretory changes followed by loss of tumor cells (9). Spontaneous regression of mammary glands during the lactation cycle was reported to be due to apoptosis (16, 17, 19) and furthermore, expression of genes such as TRPM-2, tissue transglutaminase, and poly (ADP-ribose) polymerase in these mammary glands was reported (19). A high dose of the E_2 treatment was thought to induce the same condition of the surrounding mammary carcinomas as this spontaneous regression of the lactating mammary gland. Therefore, further study on expression of these genes in the mammary carcinomas of rats with a high dose of E_2 treatment may provide valuable information in order to understand the mechanism of the apoptosis by the E_2 treatment.

Our present study showed that the incidences of mitotic cells and Br-dU positive cells were low in the regressing DMBA-induced mammary carcinomas of ovariectomized rats. Therefore, the mechanism of the inhibitory effect of ovariectomy on the growth of the mammary carcinomas was thought to be mainly related to the suppression of cell proliferation. Regression of methyl-N-nitrosourea induced rat mammary tumors after ovariectomy was reported to occur due to apoptosis (20). A discrepancy of reactions against ovariectomy between tumors in the previous study and the current study, seem to be due to differences in the biological characteristics of mammary tumors, because each tumor was induced by a different method.

Acknowledgments: We are grateful to T. Kodama and T. Nitanda for their technical assistance.

References

- McGuire WL. Steroid hormone receptors in breast cancer treatment strategy. *Recent Prog Horm Res* 1980; **36**: 135-156.

2. Nomura Y, Kobayashi S, Takatou O, Sugano H, Matsumoto K, and McGuire WI. Estrogen receptor and endocrine responsiveness in Japanese versus American breast cancer patients. *Cancer Res* 1977; **37**: 106-110.
3. Bodwin JS, Hirayama PH, Rego JA, and Cho-Chung YS. Regression of hormone-dependent mammary tumors in Sprague-Dawley rats as result of Tamoxifen or Pharmacologic doses of 17β -estradiol: cyclic adenosine 3', 5'-monophosphate-mediated events. *J Natl Cancer Inst* 1981; **66**: 321-326.
4. Gabriel BW, Sahai G, and Anderson, WA. Effect of high-dose estrogen on growth and steroid receptor activity in DMBA-induced mammary tumor and uteri of rats. *J Toxicol Environ Health* 1983; **11**: 437-454.
5. Huggins C and Yang NC. Induction and extinction of mammary cancer. *Science* 1962; **137**: 257-262.
6. King DT and Kennedy BJ. Combination of cyclophosphamide and estrogen therapy in DMBA-induced rat mammary cancer. *Cancer* 1971; **28**: 1202-1210.
7. Ohi Y and Yoshida H. Influence of estrogen and progesterone on the induction of mammary carcinomas by 7, 12-dimethylbenz(a)anthracene in ovariectomized rats. *Virchows Archiv B cell Pathol* 1992; **62**: 365-370.
8. 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.
9. Yoshida H, Yoshida A, Yamada M, Yamanaka K, Kitamura Y, and Matsumoto K. Regression with secretory changes ovary-independent rat mammary carcinoma by pharmacological doses of estrogen. *Jpn J Cancer Res (Gann)* 1985; **76**: 771-778.
10. Yoshida H, Suzuki M, Okugawa K, Wada S, Fukunishi R, Okamoto S, and Matsumoto K. Mammary carcinoma induced by a series of intragastric intubations of 7, 12-dimethylbenz(a)anthracene in gonadectomized female and male sprague-dawley rats. *Gann* 1982; **73**: 539-542.
11. Arends MJ, Morris RG, and Wyllie AH. Apoptosis: The role of the endonuclease. *Amer J Pathol* 1990; **136**: 593-608.
12. Gavrieli Y, Sherman Y, and Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; **119**: 493-501.
13. Schmitz G, Walten T, Seibl R, and Kessler C. Nonradioactive labelling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. *Anal Biochem* 1991; **192**: 222-231.
14. Williams GT. Programmed cell death: Apoptosis and Oncogenesis. *Cell* 1991; **65**: 1097-1098.
15. Zakeri ZF, Quaglini D, Latham T, and Lockshin RA. Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB J* 1993; **7**: 470-478.
16. Ferguson DJP and Anderson TJ. Morphological evaluation of cell turnover in relation to the menstrual cycle in the "resting" human breast. *Br J Cancer* 1981; **44**: 177-181.
17. Walker NI, Bennett RE, and Kerr JFR. Cell death by apoptosis during involution of the lactating breast in mice and rats. *Amer J Anatomy* 1989; **185**: 19-32.
18. Chapin RE, Stevens JT, Hughes CL, Kelce WR, Hess RA, and Daston GP. Symposium overviews: endocrine modulation of reproduction. *Fundam Appl Toxicol* 1996; **29**: 1-17.
19. Guenette HB, Corbeil HB, Léger J, Wong K, Mézl V, Mooibroek M, and Tenniswood M. Induction of gene expression during involution of the lactating mammary gland of the rat. *J Mol Endocrinol*. 1994; **12**(1): 47-60.
20. Beem EP, Hillebrand MJK, Benckhuijsen C, and Overdijk B. Origin of the increased activity of beta-glucuronidase in the soluble fraction of rat mammary tumors during ovariectomy-induced regression. *Cancer Res* 1987; **47**(15): 3980-3987.