Biphasic effect of short-term pregnancy hormone treatment on *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in young and old rats

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Abstract. This study examined the development of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in young and old female Lewis rats following short-term treatment with estrogen and progesterone to mimic pregnancy. Rats exposed at 4 weeks of age to MNU were treated at 6 weeks of age (early MNU/young E/P treatment) or at 24 weeks of age (early MNU/old E/P treatment) with a 21-day slow-release pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone (E/P). Other rats were exposed to MNU at 22 and again at 23 weeks of age, and were treated with E/P at 24 weeks of age (late MNU/old E/P treatment). All experimental groups were compared with respective MNU-exposed age-matched E/P-untreated rats. Overt mammary carcinomas (≥1 cm in diameter) that were positive for hormone receptors were reduced in young E/P-treated rats, while hormone receptor-negative overt mammary carcinomas increased in old E/P-treated rats. The rate of development of small-sized mammary carcinoma (<1 cm in diameter) was similar in early MNU/young E/P-treated and late MNU/old E/P-treated groups, but higher in early MNU/old E/P-treated rats compared with respective E/P-untreated rats. At the termination of the experiment, normal mammary gland architecture had not been influenced by E/P treatment, although E/P treatment of older rats caused an increase in proliferating cell nuclear antigen (PCNA) labeling of the mammary tissue. Thus, the impact of short-term E/P treatment on MNU-induced rat mammary carcinogenesis is age-dependent and shows biphasic effects; the development of hormone-dependent overt mammary carcinomas was reduced in young rats but the development of hormone-independent overt mammary carcinomas increased in older rats. The enhanced outgrowth of hormone-independent overt mammary carcinomas by E/P treatment in old age is due to accelerated cell proliferation at the promotion/progression phase of mammary carcinogenesis. Age at short-term E/P treatment is crucial for breast cancer control.

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Introduction

Epidemiologically, full-term pregnancy at a younger age is associated with a lower risk of breast cancer compared with nulliparous women (1-5). However, parity protection becomes weaker with increasing age, and women bearing a first child over age 30 show a higher breast cancer risk than nulliparous women. In humans, the cause of breast cancer is highly complex and is usually unknown, but the carcinogenic effects of accidental or therapeutic exposure to radiation is well documented. Breast cancer risk in women exposed to radiation at a young age is reduced by a subsequent full-term pregnancy while young, but risk is increased by nulliparity or a late first pregnancy (6). Parity protection is also seen in rats and mice (7,8); pregnancy at a younger age is highly protective against mammary cancer while pregnancy in older animals is less effective (9).

Parity protection against carcinogen-induced mammary cancer in rodents can be reproduced by short-term treatment (3 weeks; approximately equivalent to the gestation period of rodents or shorter) with the pregnancy hormones estrogen and progesterone before (10-13) or shortly after exposure (11,14). Different doses and methods of hormone administration have been reported (9,15). The key to success in breast cancer protection is to achieve pregnancy levels of estrogen and progesterone for a short duration (9,15,16). Administration of pregnancy hormone to nulliparous women may be a useful strategy for protection against breast cancer. However, the age at which it is administered may be crucial.

In humans, the timing of initiation in breast epithelial cells by carcinogenic stimuli is usually unknown. However, epidemiological studies indicate that sensitivity to radiation is highest when exposure occurs during childhood (6). A single injection of the mammotrophic chemical carcinogen *N*-methyl-*N*-nitrosourea (MNU) can effectively induce mammary carcinomas in susceptible strains of rats; female prepubertal rats (≤1 month of age) are more susceptible than adolescent rats (2 months of age), while middle-aged (6-8 months of age) rats are rather resistant (17,18). Rats exposed

to the carcinogen during the adolescent period and treated at an older age (6 months) with estrogen and progesterone for a short duration develop more mammary carcinomas (19). Rat mammary glands in this experimental setting, as well as the breasts of older women, are more likely to contain transformed malignant cells than the breasts of younger females. This is due to a time (age)-dependent increase in DNA damage and mutations, thus estrogen and progesterone might be involved in stimulating and accelerating the growth of these cells. However, to better mimic humans, the prepubertal period of rats may be more suitable for carcinogenic insult. In humans, the time of carcinogenic stimuli may also occur late in life. Taken together, mammary carcinogenesis in old rats after carcinogen exposure and treatment in the prepubertal period or late in life with pregnancy hormones needs to be evaluated.

The aim of this study was to evaluate the timing of pregnancy hormone treatment and mammary carcinogenesis in relation to age at carcinogen exposure. In the present study, MNU was administered to Lewis rats at 4 weeks of age (prepuberty) for early exposure, or at 22 and 23 weeks of age (middle age) for late exposure. Hormone treatment in young rats was started at puberty, at 6 weeks of age, which is when the vaginal opening is usually seen. Hormone treatment in older rats was started at 24 weeks, an age when no major remodeling of mammary glands takes place in virgin rats (19). A 21-day slow-release pellet containing estrogen and progesterone (E/P) was used to administer the hormones. The rats were divided into six experimental groups. The early MNU/young E/P group of rats, exposed to MNU at 4 weeks (prepuberty) and administered E/P treatment soon afterward, was considered the internal positive control for mammary cancer suppression. The early MNU/no E/P group served as its control. The other experimental groups were early MNU/old E/P and late MNU/old E/P, and their respective age-matched E/P-untreated controls.

Materials and methods

Animals. Lewis rats were purchased from Charles River Japan (Atsugi, Japan). The animals were housed in a plastic cage with paper bedding (Paper Clean, SLC, Hamamatsu, Japan), 3-4 rats per cage, in a temperature- (22±2˚C) and humidity-(60±10%) controlled animal room under a 12-h light/dark cycle. They were fed a commercial pellet diet (CMF; Oriental Yeast, Chiba, Japan) and had *ad libitum* access to water throughout the experiment. All procedures concerning experimental animals were approved by the Animal Experimentation Committee of Kansai Medical University.

Experimental procedure. Rats were injected intraperitoneally (i.p.) with 50 mg/kg MNU (Groups 1 and 2) or 15 mg/kg MNU (Groups 3 and 4) at 4 weeks of age, or with 50 mg/kg MNU at 22 and again at 23 weeks of age (Groups 5 and 6). The MNU was purchased from Chem Service (West Chester, PA, USA), stored at -20˚C in the dark and dissolved in physiological saline containing 0.05% acetic acid immediately prior to injection. The slow-release E/P pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone (Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously (subQ) in the back at 6 weeks of age (Group 1) or 24 weeks of age (Groups 3 and 5). This pellet provides a steady release of the hormones for a 21-day period. The remaining E/P-untreated rats comprised the age-matched controls (Groups 2, 4 and 6, respectively). At the time of E/P implantation, all rats including E/P-untreated controls were checked for palpable mammary tumors, and any tumor-bearing rats were excluded. The experimental protocol is shown in Fig. 1.

Mammary tumor detection and sacrifice. The rats were checked weekly by palpation for the development of mammary tumors. A rat was sacrificed when its largest mammary tumor reached a diameter of ≥1 cm. The rats that did not develop a mammary tumor ≥1 cm were sacrificed at 40 (Groups 1 and 2), 54 (Groups 3 and 4) and 52 (Groups 5 and 6) weeks of age, and the experiment was terminated.

Histological examination. Mammary tumors ≥1 cm in diameter, all six pairs of non-tumoral mammary glands and any macroscopically abnormal organs and tissues were removed at autopsy and fixed in neutral buffered formalin. In addition, half of any mammary tumor ≥1 cm and one side of the non-tumoral inguinal mammary gland were fixed in methacarn. All fixed tissues were embedded in paraffin, sectioned (thickness, 4 μm) and stained with hematoxylin and eosin (H&E) for histological examination. Mammary tumors were classified according to criteria described elsewhere (20).

Mammary carcinogenesis. One rat from Group 5 with a tumor diameter of ≥1 cm was sacrificed at 40 weeks of age. Since the tumor was a sarcoma, this rat was excluded from the study. All other mammary tumors with a diameter of ≥1 cm were histologically confirmed to be mammary carcinomas. The effect of E/P on the development of mammary carcinomas of ≥1 cm was compared with respective E/P-untreated age-matched control rats. The relative risk of the occurrence of mammary carcinomas of ≥1 cm was calculated as: (incidence of mammary carcinomas in E/P-treated rat/incidence in age-matched E/P-untreated rat) x 100. Additionally, incidence (number of rats with mammary carcinomas ≥1 cm), multiplicity (including histologically detected mammary carcinomas of any size) and latency (time from MNU exposure until the development of a mammary carcinoma ≥1 cm) were compared.

Immunohistochemistry. All mammary carcinomas ≥1 cm fixed in methacarn underwent immunohistochemical assays for the expression of estrogen receptor (ER) α and progesterone receptor (PgR). Inguinal mammary tissues fixed in methacarn and sampled at the termination of the experiment were used to analyze proliferating cell nuclear antigen (PCNA) labeling. Immunohistochemistry was performed using the labeled streptavidin-biotin (LSAB) method with an LSAB staining kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The primary antibodies used for ER, PgR and PCNA were 6F11 (Novocastra, Newcastle-upon-Tyne, UK), 10A9 (Biodesign, Saco, ME, USA) and PC10 (Novocastra), respectively. To visualize the antibodies, the antigen retrieval technique in citrate buffer

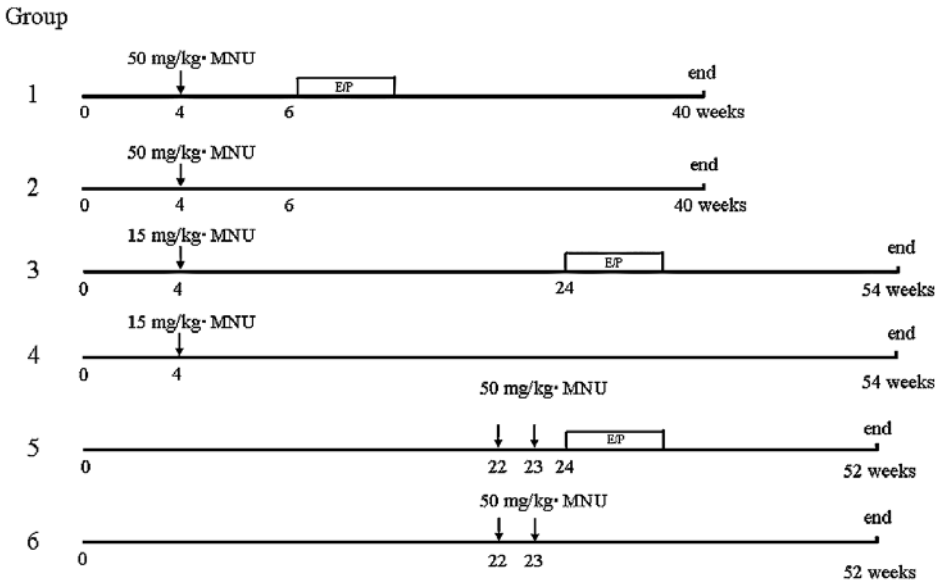

Figure 1. Schematic representation of experimental protocol. Group 1, early MNU exposure and E/P-treatment while young; Group 2, early MNU exposure and no E/P-treatment (control for Group 1); Group 3, early MNU exposure and E/P treatment while older; Group 4, early MNU exposure and no E/P-treatment (control for Group 3); Group 5, late MNU exposure and E/P treatment while older; Group 6, late MNU exposure and no E/P treatment (control for Group 5).

(pH 6.0) in a microwave oven was applied (7). Tumors in which >80% of the cells were positive for ER or PgR were considered positive for the respective receptor. To calculate the PCNA labeling index, more than 1,000 mammary glandular cells were counted in ducts and acini from more than five different areas per tissue section (7).

Statistics. All values were expressed as the mean ± standard error (SE). Final body weight, multiplicity, latency and PCNA labeling index were analyzed by the Student's t-test, and the incidence of mammary carcinomas ≥1 cm and of ER/PgR-positive carcinomas was analyzed using the χ^2 test. A probability value of $P<0.05$ was considered significant.

Results

General comments. Gains in body weight over time were smaller in E/P-treated rats (Groups 1, 3 and 5) than in their respective age-matched controls (Groups 2, 4 and 6, respectively) (Fig. 2a-c). The final mean body weight of Group 1 was significantly less than that of Group 2 ($P<0.01$). Pituitary adenomas were found in E/P-treated rats: Group 1, 15% (age 33-40 weeks, from 27 weeks after E/P); Group 3, 40% (age 45-54 weeks, from 21 weeks after E/P); Group 5, 50% (age 45-52 weeks, from 21 weeks after E/P), but not in E/P-untreated control groups. Uterine enlargement (pyometra) was observed in Group 3 (40%) and in Group 5 (17%), but not in other groups.

Mammary carcinogenesis. Relative risk for the development of mammary carcinomas ≥1 cm diameter was low in young E/P-treated rats (77%) and high in old E/P-treated rats, regardless of the time of carcinogen insult (120 and 143%) (Fig. 3). Although the incidence (% of rats with mammary carcinomas ≥1 cm) was significantly lower in young E/P-treated rats (Group 1) than in respective control rats (Group 2) (74 vs.

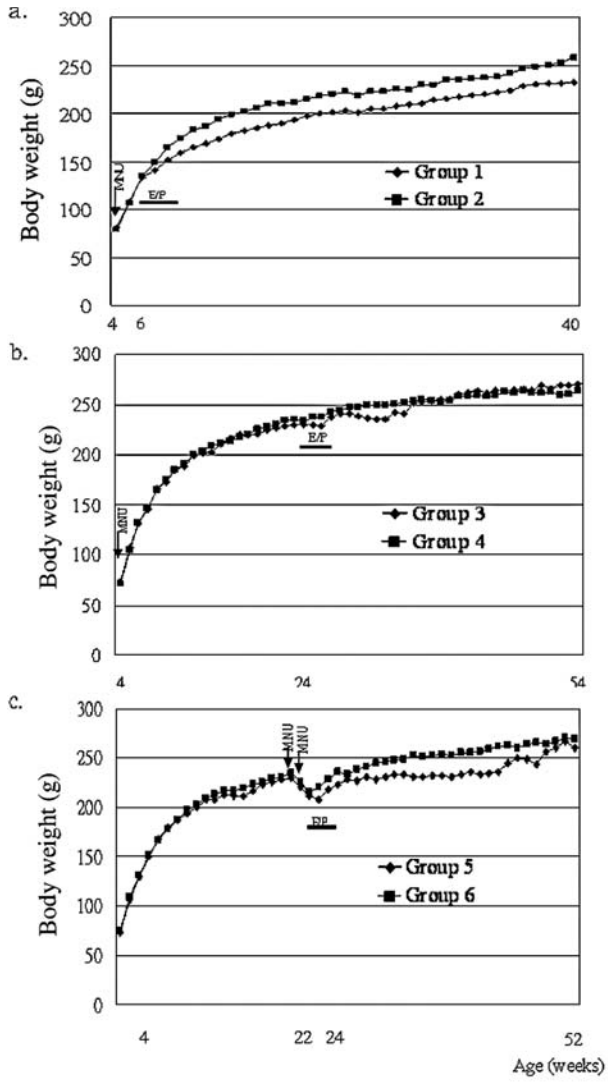

Figure 2. Body weight gain in female Lewis rats exposed to MNU and treated with or without 21-day slow-release E/P pellet.

Table I. Effects of early MNU exposure and estrogen and progesterone treatment of young female Lewis rats on mammary carcinogenesis.

Group	E/P treatment	No. of rats examined	No. of rats with carcinomas ≥1 cm (%)	Carcinomas of any size per rat	Weeks after MNU injection
1	+	27	20 (74)[a]	5.6±0.9	25.1±1.3
2	-	27	26 (96)	5.2±0.4	26.8±1.4

Carcinogen, 50 mg/kg MNU i.p. at 4 weeks of age. E/P, subQ insertion of 21-day releasing pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone at 6 weeks of age. Termination, end at 40 weeks of age. [a]P<0.05, compared with Group 2.

Table II. Effects of early MNU exposure and estrogen and progesterone treatment of aged female Lewis rats on mammary carcinogenesis.

Group	E/P treatment	No. of rats examined	No. of rats with carcinomas ≥1 cm (%)	Carcinomas of any size per rat	Weeks after MNU injection
3	+	15	6 (40)	1.1±0.3	34.0±2.2
4	-	15	5 (33)	0.5±2.2	42.8±4.6

Carcinogen, 15 mg/kg MNU i.p. at 4 weeks of age. E/P, subQ insertion of 21-day releasing pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone at 24 weeks of age. Termination, end at 54 weeks of age.

Table III. Effects of late MNU exposure and estrogen and progesterone treatment of aged female Lewis rats on mammary carcinogenesis.

Group	E/P treatment	No. of rats examined	No. of rats with carcinomas ≥1 cm (%)	Carcinomas of any size per rat	Weeks after MNU injection
5	+	12	10 (83)	2.2±0.4	22.5±2.4
6	-	12	7 (55)	2.2±0.6	27.6±1.1

Carcinogen, 50 mg/kg MNU i.p. at 22 and 23 weeks of age. E/P, subQ insertion of 21-day releasing pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone at 24 weeks of age. Termination, end at 52 weeks of age.

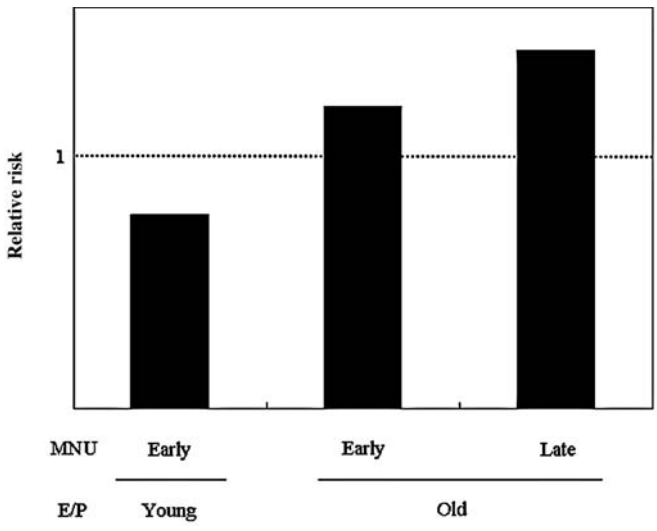

Figure 3. Relative risk of developing mammary carcinomas ≥1 cm in young and old Lewis rats after E/P treatment compared with E/P-untreated controls. Early MNU/young E/P: 50 mg/kg MNU i.p. at 4 weeks of age and E/P pellet subQ at 6 weeks of age. Early MNU/old E/P: 15 mg/kg MNU i.p. at 4 weeks of age and E/P pellet subQ at 24 weeks of age. Late MNU/old E/P: 50 mg/kg MNU i.p. each at 22 and again at 23 weeks of age and E/P pellet subQ at 24 weeks of age.

96%), multiplicity (the number of mammary carcinomas of any size per rat) and latency (weeks after MNU injection until sacrifice) were not significantly different (Table I). In older E/P-treated rats exposed to MNU early in life (Group 3), both the incidence of mammary carcinomas ≥1 cm and multiplicity increased, and time of latency was shorter. However, none of the indices were significantly different

Table IV. Effects of estrogen and progesterone treatment of female Lewis rats on ER and PgR expression in MNU-induced mammary carcinomas ≥1 cm.

Group	E/P treatment	No. of carcinomas examined	No. of ER-positive carcinomas (%)	No. of PgR-positive carcinomas (%)
1	+	32	5 (16)[a]	6 (19)[a]
2	-	52	48 (92)	34 (65)
3	+	9	2 (22)	7 (78)
4	-	6	6 (100)	4 (67)
5	+	13	3 (23)	5 (38)
6	-	9	8 (89)	4 (44)

[a]P<0.01, compared with respective control.

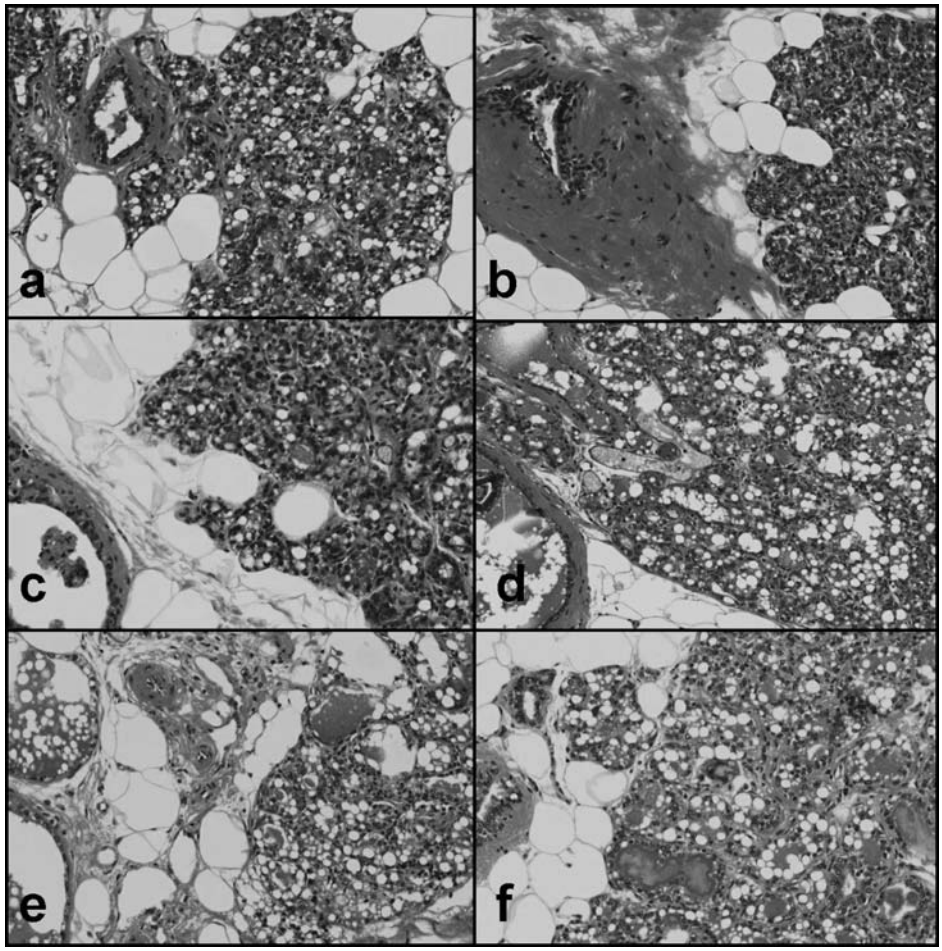

Figure 4. Mammary glands of MNU-exposed female Lewis rats treated or untreated with 21-day slow-release E/P pellet. MNU exposure was at 4 weeks of age and E/P treatment began at 6 weeks of age (a) or were E/P-untreated (b), and sampled at 40 weeks of age. MNU exposure was at 4 weeks of age and E/P treatment began at 24 weeks of age (c) or were E/P-untreated (d), and sampled at 54 weeks of age. MNU exposure was at 22 and again at 23 weeks of age, and E/P treatment began at 24 weeks of age (e) or were E/P-untreated (f), and sampled at 52 weeks of age. Short-term E/P treatment caused no adverse effect on mammary gland differentiation compared with E/P-untreated controls.

from the respective control (Group 4) (Table II). In older E/P-treated rats exposed to MNU immediately before E/P treatment, the incidence of mammary carcinomas ≥1 cm increased and latency was shorter compared with the respective control group (Group 6), while multiplicity was comparable (Table III).

Receptor status of mammary carcinomas ≥1 cm. In the E/P-treated groups (Groups 1, 3 and 5), fewer ER- and/or PgR-positive carcinomas developed compared with the respective control groups (Groups 2, 4 and 6, respectively). Significantly fewer ER- and PgR-positive carcinomas were noted in young E/P-treated rats, and larger numbers of ER- and PgR-neg-

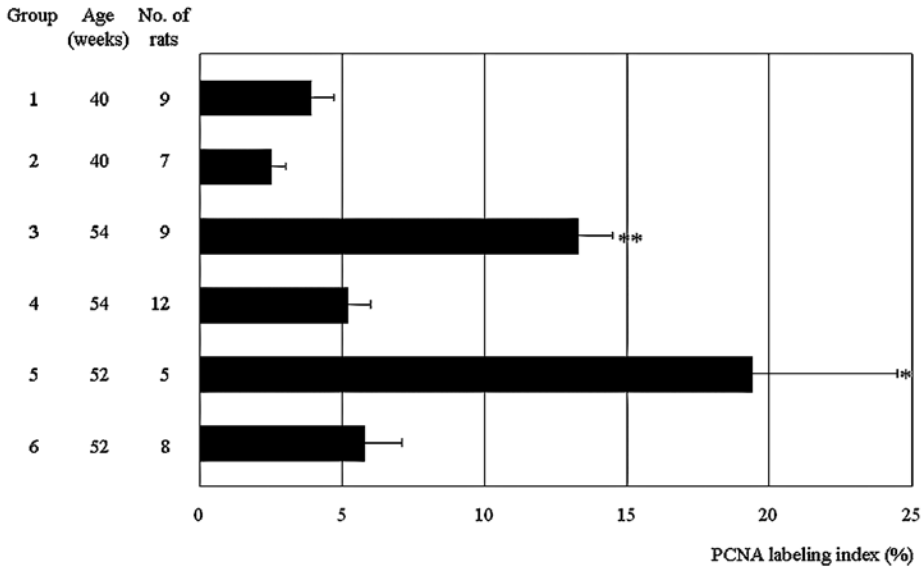

Figure 5. PCNA labeling indices of non-tumoral inguinal mammary epithelial cells of E/P-treated and -untreated rats exposed to MNU. $^{**}P<0.01$ and $^{*}P<0.05$, compared with respective controls.

ative carcinomas tended to develop in older E/P-treated rats (Table IV).

Normal mammary gland structure and PCNA labeling index. At the termination of the experiment, mammary glands of E/P-treated and E/P-untreated control rats were all composed of developed, secretory alveolar cells and were comparable in structure (Fig. 4). The development of pituitary adenoma and pyometra was not related to the grade of mammary gland differentiation or mammary cancer yields. However, at the termination of the experiment, normal mammary glands from older rats receiving E/P treatment (Groups 3 and 5) exhibited a significantly higher PCNA labeling index compared with their respective controls (Groups 4 and 6, respectively), while glands from young E/P-treated rats showed no such trend (Fig. 5).

Discussion

Women who have undergone a first full-term pregnancy before 20 years of age have a 50% reduced lifetime risk of developing breast cancer when compared with nulliparous women (1). Short-term treatment of young adult rats (≤3 months old) with estrogen and progesterone mimics the pregnancy milieu and is highly effective in suppressing mammary carcinomas in carcinogen-exposed rats (11,14,21,22). The E/P pellet we used elevates serum 17ß-estradiol and progesterone levels 2 weeks after implantation to levels comparable to pregnancy, which then drop to control levels 8 weeks after implantation (21). In this way, the present E/P regimen effectively produces the hormonal milieu of pregnancy for a short duration. In the present study, the relative risk of developing overt mammary carcinoma (≥1 cm in diameter) in young animals following carcinogen insult was reduced to 77% after E/P treatment. Parous rats (7) and pregnancy hormone-treated rats (16) develop small-sized (<1 cm in diameter) carcinomas at a rate comparable to age-matched E/P-untreated rats. However, these carcinomas do not further progress to form overt mammary carcinomas. Again, the present study showed that the yield of mammary carcinomas of any size (multiplicity) did not decrease. These data indicate that the growth of mammary cancer was blocked in the promotion and/or the progression phase after E/P treatment of young rats.

Early full-term pregnancy reduces breast cancer risk, but a transient increase in breast cancer risk is noted in women who are at least 25 years old for 3-5 years after a pregnancy (4,23). Pregnancy increases the lifetime risk of breast cancer in women who are over 30 at the time of first pregnancy (1-5). In mice administered mammary epithelial transplants that have a homozygous deletion in the mouse *p53* gene, the protective effect of pregnancy hormones was preserved when administered to older mice (age 23 to 25 weeks) as well as to young mice (age 5 to 7 weeks) (24), indicating that the timing of treatment was not crucial in this system model. However, comparable to humans, E/P treatment in older rats elevated the relative risk to 120-143% in the present rat model. In agreement with a previous report (19), mammary carcinogenesis increased in rats exposed to MNU early in life and treated with E/P for a short duration later in life (from 24 weeks of age), including the development of small-sized carcinomas. When the period from initiation (MNU exposure) to E/P treatment is long, mammary glands may house transformed cells that develop into small-sized carcinomas in the presence of pregnancy hormones. In contrast, the development of small-sized carcinomas was not increased in rats exposed to MNU late in life (at 22 or 23 weeks of age) and immediately given E/P treatment (from 24 weeks of age). Since initiated cells may exist, the time needed for transformed cells to accumulate before treatment with pregnancy hormones may not have been sufficient. However, in contrast with E/P treatment in young rats, and regardless of whether the carcinogenic insult was early or late in life, pregnancy hormone treatment in older rats increased the development of overt mammary carcinomas.

The short-term E/P treatment described here caused pituitary adenomas and/or pyometra that may influence and/or be influenced by the hormonal milieu. Pituitary adenomas are spontaneously seen in Lewis rats (25), and high doses of estrogen produce prolactin-secreting pituitary adenomas (26). However, no alteration in the mammary glandular architecture was noted in rats with adenoma and/or pyometra at the termination of the experiment. In addition, E/P treatment did not alter mammary gland morphology compared with E/P-untreated rats, and led to apparent normal physiological aging of the Lewis rat mammary gland (27). Thus, structural differentiation of the mammary gland per se does not seem to influence mammary carcinogenesis. Elevated PCNA labeling was noted in non-tumor cells of the mammary gland in older E/P-treated rats. E/P treatment of older rats not only accelerates the growth of normal cells, but also stimulates the growth of initiated cells and/or malignant-transformed cells, resulting in a high incidence of overt mammary carcinomas.

Consistent with our previous findings (7), the majority (>80%) of the rats in this study not treated with E/P developed ER-positive carcinomas. Parity-beneficial effects seem to be confined to ER-positive/PgR-positive breast cancer, but not to ER-negative/PgR-negative breast cancer (28); parity has been associated with an increased risk of developing ER-negative/PgR-negative breast cancer (29). Overt mammary carcinomas that are positive for ER and/or PgR were significantly suppressed in young E/P-treated rats. Furthermore, in agreement with a previous report (19), carcinomas that developed in the older E/P-treated rats were predominantly ER-negative. The importance of stroma in mammary carcinogenesis has been suggested (30), and there is experimental evidence suggesting that estrogen promotes the outgrowth of ER-negative cancers by stimulating stromal cells distinct from breast epithelial cells (31). Older rats treated with E/P preferentially developed ER-negative carcinomas by accelerating cell proliferation, stimulating the promotion /progression phase of mammary carcinogenesis and increasing the development of overt mammary carcinomas. Molecular analysis of young parous mammary gland showed that differentiation-related genes are upregulated while growth-related genes are downregulated (32), and that carcinogen-induced cell proliferation is suppressed (32,33). Further molecular studies are needed to elucidate the mechanism by which pregnancy hormone treatment accelerates breast cancer development in older rats.

In the present study, the development of carcinogen-induced overt mammary cancer decreased in young rats but increased in older rats following short-term E/P treatment. Age is therefore a crucial factor when considering short-term E/P treatment for breast cancer control.

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