Effects of Different Durations of Estrogen and Progesterone Treatment on Development of $N$-methyl-$N$-nitrosourea-induced Mammary Carcinomas in Female Lewis Rats

TAKASHI YURI, REIKO TSUKAMOTO, NORIHISA UEHARA, YOICHIRO MATSUOKA and AIRO TSUBURA

Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

Abstract. Background: There have been no precise evaluations of the effects of different durations of exposure to estrogen and progesterone pregnancy levels on mammary carcinogenesis risk. We examined such effects on the development of $N$-methyl-$N$-nitrosourea (MNU)-induced rat mammary carcinoma. Materials and Methods: Female Lewis rats were administered a single intraperitoneal injection of 50 mg/kg MNU at 28 days of age, and then were either left hormone-untreated (control group), or underwent subcutaneous implantation of a 21-day release pellet containing 0.5 mg 17β-estradiol and 32.5 mg progesterone (E/P pellet) at 42 days of age. The pellet was either replaced every 3 to 4 weeks throughout the experimental period (long-term E/P group), or was implanted only once (short-term E/P group). Circulating 17β-estradiol and progesterone levels in the serum, and expression of estrogen receptor (ER) α and progesterone receptor (PgR) in the normal mammary gland were measured. The rats were sacrificed when they developed a mammary tumor with a diameter of ≥1 cm, or when they reached the age of 29 weeks. Results: In rats implanted with a single E/P pellet, circulating 17β-estradiol and progesterone levels were significantly elevated 2 weeks after implantation, but returned to control levels 8 weeks after implantation; 17β-estradiol transiently reached pregnancy levels. In normal mammary glands of rats sacrificed at 29 weeks of age, both long- and short-term E/P treatment decreased the percentage of ERα- and PgR-positive cells. Rats that received long- or short-term E/P treatment had a decreased incidence of mammary carcinoma with a diameter of ≥1 cm, compared to control rats. However, when histologically detected microcarcinomas (diameter <1 cm) were included for comparison, the E/P-treated groups exhibited an abrupt increase in the number of microcarcinomas from 22 to 25 weeks after MNU injection. Although short-term E/P treatment significantly suppressed mammary carcinomas of all sizes, long-term E/P treatment had no cancer-suppressing effect. Conclusion: The duration of E/P treatment is an essential factor for the suppression of mammary carcinogenesis.

Early age at first full-term pregnancy inversely correlates with breast cancer risk (1-4); it is the only normal physiological condition that consistently protects against breast cancer in all ethnic groups and countries, without known adverse effects. The protective effect of pregnancy is also seen in rats and mice (5, 6). A full-term pregnancy or pregnancy and lactation before or soon after carcinogen exposure significantly reduces overall mammary cancer incidence and multiplicity in female rats (5-8). The protective effect of pregnancy can be observed even when the carcinogen is administered for a long period (100-130 days) after the delivery, indicating that parity-induced protection is a long-lasting phenomenon (9). The protective effects of pregnancy against mammary cancer can be mimicked in the rat by short-term treatment (21 days or slightly longer; gestation period of the rat is 21 days) with estrogen and progesterone. Short-term treatment with estrogen plus progesterone before, during or after carcinogen exposure is highly effective in suppressing mammary carcinogenesis (10-17). Mimicking the hormonal state of pregnancy by administering estrogen and progesterone may be a safe and effective way of decreasing breast cancer risk in nulliparous women.

Pregnancy and lactation decrease breast cancer risk. However, pregnancy has a dual effect on the risk of breast cancer: it reduces the risk in later years, but transiently increases the risk after childbirth (3, 18). In rats, while overall incidence of chemically-induced rat mammary
cancer is reduced at the end of the experiment, the risk of chemically-induced carcinomas is transiently accelerated during pregnancy (7). Therefore, prolonged exposure to the hormonal environment of pregnancy may increase the overall risk of mammary cancer, probably due to a growth-enhancing effect of the estrogen and/or progesterone. However, there have been no precise evaluations of the effects of different durations of exposure to pregnancy levels of estrogen and progesterone on the risk of mammary carcinoma. In the present study, the effects of short- and long-term estrogen and progesterone exposure on the development of N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas were examined in female Lewis rats, with hormone-untreated control rats included for comparison.

Materials and Methods

Animals. Three-week-old female Lewis rats were purchased from Charles River Japan (Atsugi, Japan). The animals were housed in a temperature- and humidity-controlled animal room in plastic cages, 3 to 4 rats per cage, with wood-chip bedding, at 22±2°C and 60±10% humidity, under a 12-h light/dark cycle. They had free access to a commercial pellet diet (CMF; Oriental Yeast, Chiba, Japan) and water throughout the experiment. All experimental animal procedures were approved by the Animal Experimentation Committee of Kansai Medical University, Japan.

Hormone and carcinogen treatments. At 28 days of age, rats were intraperitoneally injected with 50 mg/kg MNU. The MNU was purchased from Nacalai Tesque (Kyoto, Japan), stocked at −20°C in the dark, and dissolved in physiological saline containing 0.05% acetic acid immediately before the injection.

In humans, the time of carcinogenic insult (initiation of breast cancer) is unknown, but may happen early in life. MNU is a direct-acting carcinogen and can induce mammary carcinomas in sexually immature female rats (19). Thus, the rats in the present study were injected with MNU at 28 days of age, when they were still sexually immature; few rats were not injected with MNU.

The MNU-treated rats were then divided into 3 groups: control group, short-term E/P group, and long-term E/P group. The control group received no hormone treatment. In the short-term E/P group and long-term E/P group, at 42 days of age, a pellet containing 0.5 mg 17β-estradiol and 32.5 mg progesterone (E/P pellet; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously into the back of each rat. The E/P pellet steadily releases the hormones over a 21-day period. The first E/P pellet was implanted at 42 days of age because in Lewis rats, puberty onset (vaginal opening) occurs at 40 to 43 days of age (average, 40.9±0.4 days; (20)). In the short-term E/P group, a single pellet was implanted into each rat. In the long-term E/P group, every 3 to 4 weeks beginning at 42 days of age, a new E/P pellet was implanted into each rat to replace the previous E/P pellet.

Circulating hormone levels. In order to measure hormone levels, rats were sacrificed by anesthesia with ether, and blood was collected via cardiac puncture. In the short-term E/P group, rats were sacrificed 2, 8 and 23 weeks after the single pellet implantation (8, 14 and 29 weeks of age, respectively). In the long-term E/P group, rats were sacrificed 23 weeks after the first pellet implantation (29 weeks of age). The serum concentration of 17β-estradiol and progesterone were measured using radioimmunoassay kits (Diagnostic Products, Los Angeles, CA, USA) according to manufacturer’s instructions, and compared with E/P-untreated controls.

Mammary tumor detection and latency. Once per week, rats were weighed and checked for mammary tumors by palpation. Rats were sacrificed when their largest mammary tumor reached a diameter of ≥1 cm; the tumors were removed at autopsy and used for the histological examination described below. Latency was defined as the time interval between MNU injection and the point at which the largest mammary tumor reached a diameter of ≥1 cm. Rats that never developed a mammary tumor with a diameter of ≥1 cm were sacrificed 25 weeks after MNU injection (29 weeks of age); their (normal) mammary glands were removed at autopsy and used for the histological examination and hormone receptor assays described below.

Histological examination. The mammary tumors and normal mammary glands obtained at autopsy were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin for histological examination. Both mammary tumors with a diameter of ≥1 cm and microtumors (diameter <1 cm) were examined. All tumors were classified as carcinoma or fibroadenoma.

Hormone receptors in normal mammary glands. The expression of hormone receptors in normal mammary glands of rats sacrificed at 29 weeks of age was evaluated. The assays for estrogen receptor (ER) α and progesterone receptor (PgR) were performed using the labeled streptavidin-biotin (LSAB) method with an LSAB staining kit (DakoCytomation, Carpenteria, CA, USA) according to the manufacturer’s instructions. The following antibodies against ERα and PgR were used: 1D5 and 10A9, respectively (Immunootech., Marseille, France). For the visualization of antibodies, the antigen retrieval technique was performed using a citrate buffer (pH 6.0) and a microwave oven. Cells in ducts and lobules were counted, and the percentages of ERα- and PgR-positive cells were calculated.

Statistical analysis. All results are expressed as mean±standard error (SE). Tumor incidence was analyzed using the Mantel-Cox log-rank test. The numbers of rats with mammary tumors were analyzed for independence using the Chi-square test. For all other data, after assurance of homogeneity of variance, analysis was performed using the unpaired t-test or Mann-Whitney U-test. A probability value of p<0.01 was considered to indicate statistical significance.

Results

Circulating hormone levels. In the short-term E/P group, circulating 17β-estradiol and progesterone levels were elevated at 8 weeks of age (2 weeks after single E/P pellet implantation), but returned to control levels at 14 weeks of age (8 weeks after implantation) (Figure 1). In previous studies using Long-Evans and Sprague-Dawley rats, serum estrogen levels during pregnancy have been found to range from 55 to
In the short-term E/P group, circulating estrogen transiently reached pregnancy levels. At 29 weeks of age, hormone levels in the short-term E/P group were comparable to control levels. In contrast, at 29 weeks of age in the long-term E/P group, circulating 17β-estradiol was at pregnancy levels, whereas progesterone levels were comparable to control levels; the final E/P pellet was implanted 4 weeks before the measurement of hormone levels.

Body weight. Seven rats in the long-term E/P group, and 1 rat in the short-term E/P group died; these 8 rats were excluded from analysis of body weight. The body weight curves are shown in Figure 2. Body weight gain was lower in the 2 E/P groups than in the control group (the long-term E/P group had the lowest body weight gain), but there were not significant differences in final body weight among the groups.

Structure of normal mammary glands. In the short-term E/P group, at 2 weeks after E/P pellet implantation (8 weeks of age), we observed rapid proliferation and differentiation of mammary glands; i.e., a structure comparable to that of mammary glands in late pregnancy. In the long-term E/P group, at 23 weeks after E/P pellet implantation (29 weeks of age), mammary glands were more differentiated than those of age-matched controls, and had large ducts and alveoli filled with milk. In rats of the short-term E/P group sacrificed at 29 weeks of age, mammary glands were moderately differentiated, compared with age-matched controls. In all 3 groups, mammary glandular structures reflected the respective hormonal environments.

Hormone receptors in normal mammary glands. At 29 weeks of age, the percentage of ERα- and PgR-positive cells in mammary glands of the control, long-term E/P, and short-term-E/P groups was 23.3% and 33.5%, 7.3% and 17.3%, and 7.7% and 8.8%, respectively (Figure 3). In the long- and short-term E/P groups, the percentage of both ERα- and PgR-positive cells was lower, compared to controls.

Mammary tumorigenesis. The control group had a 95% incidence of mammary tumors with a diameter of ≥1 cm, with an average latency of 17.4 weeks (Figure 4). In the long- and short-term E/P groups, the incidence and latency of mammary tumors with a diameter of ≥1 cm were 36% and 21.6 weeks, and 21% and 22.3 weeks, respectively. Both E/P groups had a significantly lower incidence of mammary tumors than the control group (p<0.01, respectively). The E/P groups also had a somewhat longer latency than the control group, but there were no significant differences in latency between groups. All mammary tumors with a
Figure 2. Body weight gain of female Lewis rats injected intraperitoneally with N-methyl-N-nitrosourea (MNU) at 4 weeks of age and subcutaneously implanted with estrogen and progesterone (E/P) pellet for different durations starting at 6 weeks of age.

Figure 3. Effects of estrogen and progesterone (E/P) treatment on the percentage of estrogen receptor (ER)- and progesterone receptor (PgR)-positive cells in normal mammary glands of female Lewis rats at 29 weeks of age. Each column represents data for 4-6 rats. *p <0.05 and **p<0.01, compared with E/P-untreated controls.
diameter of ≥1 cm were carcinomas. We evaluated mammary carcinomas with a diameter of ≥1 cm, mammary carcinomas of all sizes (including histologically detected microcarcinomas), and benign tumors (Table I). The E/P groups had a significantly lower incidence and number of mammary carcinomas with a diameter of ≥1 cm per rat than the control group \((p<0.01)\). However, in both E/P groups, the number of microcarcinomas abruptly increased from 23 to 25 weeks after MNU injection, causing an increase in the number of carcinomas of all sizes per rat (Figure 5). The short-term E/P group had a significantly lower number of mammary carcinomas of all sizes per rat than the control group, but there was no significant difference in the number of mammary carcinomas of all sizes per rat between the long-term E/P group and the control group. In addition, the long-term E/P group had a significantly greater number of rats with fibroadenomas than the other groups (Table I).

**Discussion**

Full-term pregnancy markedly reduces the risk of chemically-induced mammary carcinomas in rats (5, 7, 9), and short-term treatment with estrogen and progesterone can mimic the protective effect of pregnancy (10-17). In the present study, consistent with previous reports (15, 24), short-term E/P treatment of Lewis rats transiently elevated circulating estrogen and progesterone to levels comparable to pregnancy levels, and effectively suppressed the incidence of carcinomas of all sizes (gross and microscopic). However, when microcarcinomas were included in the analysis, long-term E/P treatment did not suppress the incidence of carcinomas. Although there were no significant differences in final body weight, the long- and short-term E/P groups tended to have slower body weight gain than the controls. The long-term E/P group had the slowest body weight gain. Thus, body weight *per se* does not seem to play a role in mammary carcinogenesis.

The available evidence clearly shows that ovariectomy lowers the risk of mammary carcinomas in humans (1) and rats (11), indicating that physiological levels of ovarian steroids (estrogen and progesterone) are generally required for mammary carcinogenesis; exogenous ovarian steroids accelerate mammary carcinogenesis in female rats with low levels of female sex hormones. In contrast, rats in which levels of estrogen and progesterone are raised to pregnancy levels for a short duration (as short as 1-2 weeks) have lower mammary cancer risk than rats with normal non-pregnancy levels of estrogen and progesterone (25). Although it is
more effective to combine estrogen with progesterone, short-term treatment with pregnancy levels of estrogen alone can reduce the risk of mammary carcinogenesis (15). However, studies indicate that earliness of menarche and the total length of ovarian steroid exposure positively correlate with risk of breast cancer, suggesting that long-term exposure to pregnancy levels of estrogen can cancel out the mammary-cancer-inhibiting effects of short-term exposure or even accelerate mammary carcinogenesis.

In Sprague-Dawley rats, a 12-week exposure to pregnancy levels of estrogen was found to completely abolish development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced grossly visible mammary carcinomas (26). In growth hormone (GH)-deficient spontaneous dwarf rats (derived from the Sprague-Dawley strain), which are refractory to chemical induction of mammary carcinomas, a 20-week exposure to GH induced mammary carcinogenesis, but treatment with a combination of estrogen, progesterone and GH abolished the GH-induced increase in incidence of MNU-induced grossly visible mammary carcinomas (27); small numbers of grossly visible carcinomas began to appear after 20 weeks of exposure to estrogen and

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**Table I. Effects of duration of estrogen and progesterone treatment on histologically detected mammary tumors in MNU-injected female Lewis rats.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of rats ≥1 cm (%)</th>
<th>No. of rats ≥1 cm per rat</th>
<th>No. of rats with any carcinoma (%)</th>
<th>No. of any carcinoma per rat</th>
<th>No. of rats with fibroadenoma (%)</th>
<th>No. of fibroadenoma per rat</th>
<th>Latency (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E/P-untreated</td>
<td>20</td>
<td>19 (95)</td>
<td>1.5±0.2</td>
<td>20 (100)</td>
<td>3.3±0.4</td>
<td>0 (0)</td>
<td>0</td>
<td>17.4±1.2</td>
</tr>
<tr>
<td>Long-term E/P</td>
<td>14</td>
<td>5 (36)**</td>
<td>0.4±0.1**</td>
<td>9 (64)**</td>
<td>3.4±1.4</td>
<td>5 (36)**,#</td>
<td>0.4±0.2</td>
<td>21.6±1.7</td>
</tr>
<tr>
<td>Short-term E/P</td>
<td>19</td>
<td>4 (21)**</td>
<td>0.2±0.1**</td>
<td>11 (58)**</td>
<td>1.6±0.5**</td>
<td>1 (5)</td>
<td>0.1±0.1</td>
<td>22.3±1.7</td>
</tr>
</tbody>
</table>

*p values <0.05 and **<0.01, compared with E/P-untreated group; #p<0.05 compared with short-term E/P group.

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**Figure 5. Effects of estrogen and progesterone (E/P) treatment on the cumulative number of N-methyl-N-nitrosourea (MNU)-induced carcinomas of all sizes per rat.**

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In Sprague-Dawley rats, a 12-week exposure to pregnancy levels of estrogen was found to completely abolish development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced grossly visible mammary carcinomas (26). In growth hormone (GH)-deficient spontaneous dwarf rats (derived from the Sprague-Dawley strain), which are refractory to chemical induction of mammary carcinomas, a 20-week exposure to GH induced mammary carcinogenesis, but treatment with a combination of estrogen, progesterone and GH abolished the GH-induced increase in incidence of MNU-induced grossly visible mammary carcinomas (27); small numbers of grossly visible carcinomas began to appear after 20 weeks of exposure to estrogen and
progesterone. In the present study, in the long-term E/P group (23-week exposure), grossly visible mammary carcinomas were still suppressed; however, after >20 weeks of exposure to E/P, the number of microcarcinomas abruptly increased, and the cancer-suppressing effect of the E/P pellet disappeared. A question of clinical relevance is whether these microcarcinomas remain in a latent state or progress to grossly visible carcinomas. In a previous study using ovariectomized Sprague-Dawley rats, repeated injections of estrogen and progesterone for 28 weeks augmented the development of DMBA-induced palpable mammary carcinomas (28). Moreover, in another study, parous Sprague-Dawley rats refractory to chemically induced mammary cancer developed gross mammary carcinoma after a 33-week treatment with estrogen and progesterone (29).

These findings suggest that prolonged exposure to estrogen and/or progesterone can induce development of gross mammary carcinomas; it appears that exposure to estrogen and progesterone must be maintained for <20 weeks in order to suppress the development of grossly visible mammary carcinomas later in life. Also, in the present study, long-term (but not short-term) E/P treatment augmented the development of fibroadenomas. It has previously been found that estrogen and progesterone accelerated the growth of DMBA-induced benign mammary tumors in rats (10).

Several mechanisms have been proposed for parity-associated protection against mammary cancer, which is still not well understood (30-33). Hormone-induced mammary glandular differentiation does not seem to play a role in suppressing mammary carcinogenesis; e.g., in the present study, the long-term E/P group exhibited the greatest degree of differentiation of normal mammary gland among the 3 groups, but the number of mammary carcinomas of all sizes in this group was comparable to that of the control group. Interaction between estrogen and ERα may affect mammary carcinogenesis. In the present study, the long- and short-term E/P groups both had lower ERα expression than the control group. This present finding is consistent with previous studies of rats, in which ERα expression was found to decrease during pregnancy (34) and in parous rats (5). Thus, there appears to be no simple relationship between ERα expression and the loss of cancer-suppressing effects of long-term treatment with estrogen and progesterone. Further studies are needed to clarify the mechanisms that underlie the effects of estrogen and progesterone on mammary carcinogenesis. Short-term estrogen and progesterone treatment can suppress mammary cancer as effectively as pregnancy and ovariectomy. Thus, treatment with estrogen and progesterone may be a clinically useful strategy for the suppression of human breast cancer. However, the cancer-suppressing effect of treatment with estrogen and progesterone is lost after prolonged (>20 weeks) exposure and such long-term exposure may even promote development of gross mammary carcinomas later in life. Thus, in addition to the great potential benefits of short-term exposure to pregnancy levels of estrogen and progesterone, prolonged estrogen and progesterone treatment carries great potential risks if applied to humans.

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References


