Protective effects of pregnancy and lactation against N-methyl-N-nitrosourea-induced mammary carcinomas in female Lewis rats

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The role of parity before and after N-methyl-N-nitrosourea (MNU) treatment in protection against mammary carcinogenesis was investigated. The effect of lactation on reduction in the incidence of mammary carcinoma was also examined. Parous rats were compared with respective age-matched virgins (AMVs). Pregnancy and lactation prior to MNU exposure significantly reduced both the incidence of mammary carcinoma (22 versus 72%) and the average number of mammary carcinomas per rat (0.22 versus 0.86) and significantly prolonged the latency of the carcinomas (247 versus 215 days). Pregnancy and lactation following MNU exposure also significantly reduced both the incidence of mammary carcinoma (25 versus 94%) and the average number of mammary carcinomas per rat (0.25 versus 1.50) and significantly prolonged the latency (240 versus 155 days). Lactation showed an additive effect on the reduction in mammary cancer. Pregnancy suppressed the number of estrogen receptor (ER)- and progesterone receptor (PgR)-positive cells and lowered the cell proliferation rate in the non-tumoral mammary glands. Since the majority (>76%) of the mammary carcinomas was hormone dependent in both the parous and AMV rats, pregnancy and lactation appear to decrease the ER- and/or PgR-positive cells presumed to be the progenitors of hormone-dependent carcinomas and they lowered the cell turnover necessary for tumor promotion in parous rats, resulting in a lower mammary carcinoma yield.

Introduction

Epidemiological and clinical observations indicate that parous women have a lower incidence of breast cancer than nulliparous women and that human breast cancer is markedly influenced by the reproductive history of the individual (1,2). However, the mechanisms whereby pregnancy protects against breast cancer are unknown. Chemically induced rat mammary cancer is similarly affected by pregnancy and lactation (3). Mammary carcinogenesis is significantly inhibited when rats have completed one pregnancy prior to exposure to the carcinogen compared with age-matched virgin (AMV) rats (4–8). This protective effect was attributed to permanent structural and functional changes induced in the mammary parenchyma by the reproductive process, resulting in a lower susceptibility of epithelial cells to future carcinogenic stimuli (9,10). However, other investigators failed to detect any obvious morphological changes or altered proliferative activity in non-tumoral parous rat mammary epithelium (5,7). The mammary cancer incidence in parous rats treated with a carcinogen before pregnancy is also reduced compared with that in AMV rats (11–15). In this situation, preneoplastic cells present prior to pregnancy are speculated to be either eliminated or altered by the hormones associated with pregnancy. Thus, an inhibitory effect on mammary carcinogenesis is observed when rats have completed one pregnancy before or after carcinogen exposure. However, the mechanisms underlying this effect of pregnancy on mammary cancer are not yet clear.

Estrogen and/or progesterone are known to have important roles in the etiology of breast cancer. Hormone-dependent and -independent carcinomas occur in humans and the estrogen receptor (ER) and/or progesterone receptor (PgR) content of normal breast epithelial cells may be related to the risk of human breast cancer (16,17). The development and growth of a large proportion of N-methyl-N-nitrosourea (MNU)-induced mammary tumors in virgin rats are hormone dependent (18). ER- and/or PgR-positive cells in normal mammary glands are presumed to be the progenitors of hormone-dependent cancers (19). It is possible that the hormone environment during pregnancy may enable the differentiation of ER- and/or PgR-positive normal/preneoplastic cells to a secretory state, so that these cells do not undergo further cell division (20). ER/PgR-positive cells might thus be eliminated from the carcinogenic process and parity may therefore result in a reduction in the percentage of hormone-dependent cancers (5). Alternatively, ER/PgR-positive cells may participate in the carcinogenic process and parity may suppress mammary cancer development by decreasing the numbers of such cells in the normal mammary gland.

The topical exposure of rat mammary glands to crystalline MNU can be easily performed and results in a rapid induction and high incidence of mammary carcinomas without conspicuous side effects; approximately half of the mammary carcinomas develop at the MNU-exposed site and the rest are seen distant from the MNU-exposed gland (21). In addition, immunohistochemistry can evaluate the ER- and PgR-positive cells as well as proliferating cell nuclear antigen (PCNA)-labeled cells in tissue sections from MNU-treated rats, allowing determination of the positivity on a morphological basis (22). The present study was carried out to determine whether pregnancy and lactation in Lewis rats prior to or after topical exposure to crystalline MNU suppress the mammary carcinoma yield. Special attention was paid to the incidence of hormone-dependent cancers and to the numbers of ER- and/or PgR-positive cells, as well as the proliferating activity of these cells in the non-tumoral mammary glands of parous and AMV rats.

Materials and methods

Animals

Male and female Lewis rats were purchased from Charles River Japan (Kyoto, Japan). The animals were housed in plastic cages, 3–4 rats/cage with wood-

Abbreviations: AMV, age-matched virgin; ER, estrogen receptor; LSAB, labeled streptavidin–biotin; MNU, N-methyl-N-nitrosourea; PCNA, proliferating cell nuclear antigen; PgR, progesterone receptor.
The tumor volume was calculated using a standard formula; Tumors were measured with calipers and the change in volume was recorded.

Week. The mammary tumors were biopsied when the largest tumor reached the carcinoma incidence and hormone dependency were analyzed by the χ² test and number of mammary carcinomas per rat, latency, ER/PgR-positive rates and PCNA labeling index in autopsied non-tumoral mammary glands.

**Histological examination**

Mammary tumors and non-tumoral mammary tissues obtained at autopsy and at biopsy were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 µm and stained with hematoxylin and eosin for histological examination and for an assessment of the development of mammary glands. Serially cut sections were used for hormone receptor studies and a cell kinetic evaluation.

**Immunohistochemical examinations**

The assay of ER, PgR and PCNA was carried out by the labeled streptavidin–biotin (LSAB) method using an LSAB staining kit (Dako, Carpinteria, CA) according to the manufacturer’s instructions. Antibodies to ER and PgR, 1D5 and 10A9 (Immunotech, Marseille, France), respectively, and an antibody to PCNA, PC10 (Novocastra, Newcastle upon Tyne, UK), were used. For the visualization of antibodies, the antigen retrieval technique in a citrate buffer (pH 6.0) in a microwave oven was used (22).

**Hormone dependency**

The changes in tumor volume after ovariectomy were evaluated biologically. The tumors that regressed >50% were considered hormone dependent and the tumors that maintained a constant size or continued to grow were considered hormone independent. Immunohistochemically, biopsied tumors containing >80% ER- and/or PgR-positive cells and >70% PCNA-labeled cells and autopsied tumors containing <20% ER- and/or PgR-positive cells and <20% PCNA-labeled cells were considered hormone dependent.

**ER/PgR-positive rates and PCNA labeling index in autopsied non-tumoral mammary glands**

For the evaluation of hormone receptor-positive cells and cell proliferation rate, the left (contralateral) inguinal mammary glands from six arbitrarily selected parous rats and six AMV rats killed at the termination of the experiment (experiments I and II), respectively, were examined. Two group 4 rats and five group 5 rats (at 47 weeks of age without ovariectomy) were added for examination. More than 1000 cells were counted in ducts and lobules from five different areas per tissue section and the percentages of ER- and/or PgR-positive cells and PCNA labeling index were calculated.

**Statistical analysis**

The carcinoma incidence and hormone dependency were analyzed by the χ² test and number of mammary carcinomas per rat, latency, ER/PgR-positive rates and PCNA labeling index were analyzed by Student’s t-test.

**Results**

**General remarks**

The body weights of the parous and AMV rats at the time of MNU exposure in experiments I and II (168 and 75 days of
Parity and mammary carcinogenesis in rats

Fig. 2. Cumulative incidence of mammary carcinomas (>1 cm) induced by MNU in female Lewis rats. Parous rats (n = 18) experienced one delivery of pups (mean at 105 days of age) and lactation before MNU exposure (●); AMV rats (n = 22) (○). P, pregnancy; L, lactation (21 days). The 10 mg crystalline MNU was topically applied to the right inguinal mammary gland at 168 days of age.

Fig. 3. Cumulative incidence of mammary carcinomas (>1 cm) induced by MNU in female Lewis rats. The 10 mg crystalline MNU was topically applied to the right inguinal mammary gland at 75 days of age. Parous rats experienced one delivery (mean at 113 days of age) and lactation (n = 12) (●) or one delivery (mean at 109 days of age) without lactation (n = 17) (○). AMV rats (n = 18) (○). P, pregnancy; L, lactation.

Effect of pregnancy on mammary tumorigenesis

In experiments I and II, mammary tumors developed and were confirmed histologically. All mammary carcinomas were typical adenocarcinomas. In experiment I, the mammary carcinoma incidence in the parous rats (group 1) which received a single MNU exposure at 168 days of age after pregnancy and lactation was significantly lower and the time necessary for the cancer to reach 1 cm in diameter (latency) was significantly longer compared with the AMV rats (group 2) (Figure 2). The parous rats (group 1) exhibited a 22% incidence of mammary carcinomas, with a mean of 0.22 carcinomas/rat, and the mean time of harvest after MNU was 247 days. In contrast, the AMV rats (group 2) developed a 72% incidence (P < 0.01), with a mean of 0.86 carcinomas/rat (P < 0.01), at an average of 215 days (P < 0.05) after MNU exposure (Table I). In experiment II, compared with the AMV rats, a decrease in mammary carcinoma incidence and delay in the harvest of tumors were evident in the rats that received MNU before pregnancy and lactation or before pregnancy alone (Figure 3). The parous rats without lactation showed intermediate values. MNU exposure at 75 days of age followed by pregnancy and lactation (group 3) was compared with AMV rats (group 5). The mammary carcinoma incidence (25 versus 94%, P < 0.01) and no. of mammary carcinomas/rat (0.25 versus 1.50, P < 0.01) were significantly lower and the time necessary for the tumors to reach 1 cm in diameter (240 versus 155 days, P < 0.01) was significantly longer in the pregnant and lactation rats (group 3) compared with the AMV rats (group 5). In the pregnant rats without lactation (group 4), although not significant, the carcinoma yield and no. of carcinomas/rat were low and the latency was prolonged compared with the AMVs (group 5). Compared with the parous rats without lactation (group 4), parous rats with lactation (group 3) showed significantly lower mammary carcinoma yield and no. of carcinomas/rat (P < 0.01) and longer latency (P = 0.01).

In addition to the macroscopic carcinomas, histologically detected (<1 cm in diameter) mammary carcinomas found at autopsy were included for comparison (Table II). Again, the mammary carcinoma incidence and the number of mammary carcinomas per rat were lower in the parous rats than their respective AMVs. The final incidence and multiplicity of mammary carcinomas per rat were significant in experiment II (group 3 versus groups 4 and 5, P < 0.01), but the differences were not significant in experiment I. All other organs, including the pituitary glands were normal, even in the rats bearing mammary tumors.

Hormone dependency of MNU-induced mammary carcinomas

In the parous rats (groups 1, 3 and 4) and in the AMV rats (groups 2 and 5), most (>76%) of the mammary carcinomas, as evaluated biologically and immunohistochemically, were...
Table I. Gross mammary carcinomas (>1 cm) in Lewis rats induced by MNU

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>MNU exposure (days)</th>
<th>No. of rats</th>
<th>No. of mammary carcinoma-bearing rats (%)</th>
<th>No. of mammary carcinomas</th>
<th>Mammary carcinomas/rat (mean ± SE)</th>
<th>Latency period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1. Parous (P/L+MNU)</td>
<td>168</td>
<td>18</td>
<td>4 (22)</td>
<td>4</td>
<td>0.22 ± 0.10</td>
<td>229±257</td>
</tr>
<tr>
<td>2. AMV</td>
<td>168</td>
<td>22</td>
<td>16 (72)</td>
<td>19a</td>
<td>0.86 ± 0.14</td>
<td>126±258</td>
</tr>
<tr>
<td>II 3. Parous (MNU+P/L)</td>
<td>75</td>
<td>12</td>
<td>3 (25)</td>
<td>3b</td>
<td>0.25 ± 0.13</td>
<td>236±259</td>
</tr>
<tr>
<td>4. Parous (MNU+P)</td>
<td>75</td>
<td>17</td>
<td>13 (76)</td>
<td>17c</td>
<td>1.12 ± 0.21</td>
<td>88±259</td>
</tr>
<tr>
<td>5. AMV</td>
<td>75</td>
<td>18</td>
<td>17 (94)</td>
<td>27d</td>
<td>1.50 ± 0.26</td>
<td>82±259</td>
</tr>
</tbody>
</table>

P, pregnancy; L, lactation; AMV, age-matched virgins. Including a 4, b 2, c 2 and d 10 mammary carcinomas distant from the right inguinal gland.

Table II. Histologically determined mammary carcinomas in Lewis rats induced by MNU

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>MNU exposure (days)</th>
<th>No. of rats</th>
<th>No. of mammary carcinoma-bearing rats (%)</th>
<th>No. of mammary carcinomas</th>
<th>Mammary carcinomas/rat (mean ± SE)</th>
<th>Other mammary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1. Parous (P/L+MNU)</td>
<td>168</td>
<td>18</td>
<td>12 (67)</td>
<td>20a</td>
<td>1.11 ± 0.23</td>
<td>3 fibroadenomas 0</td>
</tr>
<tr>
<td>2. AMV</td>
<td>168</td>
<td>22</td>
<td>20 (91)</td>
<td>33b</td>
<td>1.50 ± 0.18</td>
<td>2 fibroadenomas 0</td>
</tr>
<tr>
<td>II 3. Parous (MNU+P/L)</td>
<td>75</td>
<td>12</td>
<td>6 (50)</td>
<td>8c</td>
<td>0.67 ± 0.22</td>
<td>1 fibrosarcoma 0</td>
</tr>
<tr>
<td>4. Parous (MNU+P)</td>
<td>75</td>
<td>17</td>
<td>14 (82)</td>
<td>35d</td>
<td>2.06 ± 0.37</td>
<td>0 0</td>
</tr>
<tr>
<td>5. AMV</td>
<td>75</td>
<td>18</td>
<td>17 (94)</td>
<td>54e</td>
<td>3.00 ± 0.42</td>
<td>1 adenoma 1 adrenal cortical carcinoma</td>
</tr>
</tbody>
</table>

P, pregnancy; L, lactation; AMV, age-matched virgins. Including a 1, b 5, c 2, d 3 and e 13 mammary carcinomas distant from the right inguinal gland.

Table III. Hormone responsiveness of MNU-induced mammary carcinomas in Lewis rats, determined biologically and immunohistochemically

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>MNU exposure (days)</th>
<th>Mammary carcinoma examined</th>
<th>Biologically determineda</th>
<th>Immunohistochemically determinedb</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1. Parous (P/L+MNU)</td>
<td>168</td>
<td>&gt;1 cm Total</td>
<td>2</td>
<td>2 (100) 0</td>
</tr>
<tr>
<td>2. AMV</td>
<td>168</td>
<td>&gt;1 cm Total</td>
<td>12</td>
<td>10 (83) 2 (17)</td>
</tr>
<tr>
<td>II 3. Parous (MNU+P/L)</td>
<td>75</td>
<td>&gt;1 cm Total</td>
<td>3</td>
<td>3 (100) 0</td>
</tr>
<tr>
<td>4. Parous (MNU+P)</td>
<td>75</td>
<td>&gt;1 cm Total</td>
<td>10</td>
<td>9 (90) 1 (10)</td>
</tr>
<tr>
<td>5. AMV</td>
<td>75</td>
<td>&gt;1 cm Total</td>
<td>17</td>
<td>15 (88) 2 (12)</td>
</tr>
</tbody>
</table>

P, pregnancy; L, lactation; AMV, age-matched virgins. 
aTumor growth was monitored 2–3 weeks after ovariectomy.
bExamined by ER, PgR and PCNA expression. 
aHD, hormone-dependent. 
bHID, hormone-independent.

hormone dependent and the differences in the percentages of hormone-dependent carcinomas between AMV and parous rats (group 2 versus group 1 and group 5 versus groups 3 and 4) were not significant (Table III).

Comparison of parous and AMV non-tumoral mammary glands
In experiments I and II, non-tumoral mammary glands obtained at the termination of the experiment (rats without ovariectomy) were evaluated histologically. In both experiments, the parous rats showed atrophic mammary glands compared with the respective AMVs. In both the parous and AMV rats, ER- and PgR-positive nuclei were scattered throughout the mammary glands. Reduced percentages of ER- and PgR-positive mammary luminal cells in the parous rats compared with the AMV rats were characteristic (Table IV). In experiment I, the reduction in PgR-positive cells in the parous rats compared with the AMVs was significant (P < 0.05). The PCNA labeling indices are shown in Figure 4. PCNA labeling in the parous rats was significantly lower than that in the respective AMVs (group 1 versus group 2, P < 0.01; group 3 versus group 5 and group 4 versus group 5, P < 0.05).

Discussion
The present experiments clearly show that pregnancy preceding MNU exposure and pregnancy following MNU administration were associated with significant reductions in mammary carcinomas in female Lewis rats. The age at which the animals
The effects of hormones are important for mammary carcinogenesis. In the present study, pregnancy preceding or following carcinogenic exposure resulted in fewer mammary cancers without reducing the incidence of hormone-dependent cancer; as in the virgins, most of the mammary carcinomas in the parous rats were hormone dependent. Thus, the ER/PgR-positive (normal and/or preneoplastic) cells do not seem to be eliminated from the carcinogenic process. Cells expressing ER in normal mammary epithelium are the direct progenitors of hormone-dependent cancer (19). It is noteworthy that the percentages of ER- and PgR-positive cells of underlying non-tumoral mammary glands at the termination of the present study were reduced in the parous rats, a result which paralleled their decreased incidence of mammary carcinomas. A reduction in the ER level in mammary tissue from parous animals has been noted previously (8). Since estrogens are believed to stimulate cell proliferation and since the majority of the MNU-treated mammary carcinomas observed in the present study were hormone-dependent, the ER- and/or PgR-positive cells are probably the progenitor cells of the majority of MNU-induced mammary carcinomas. In humans, ER-positive cells comprise ~7% of the total epithelial cell population (32) and PgR-positive cells account for ~19% (33). The percentages of receptor-positive cells in the non-tumoral rat mammary glands observed here are in good agreement with the values for humans.

In humans, pregnancy has a protective effect against breast cancer, which is attributed to a greater degree of differentiation along with less proliferation activity in the breast (34). In the present study, the non-tumoral mammary glands of the parous rats at the termination of the experiment showed atrophic glands with low proliferative activity. Taken together, the present results indicate that the prevention of mammary cancer by pregnancy may be based on: (i) pregnancy reducing the
number of ER- and/or PgR-positive cells in non-tumoral (normal) mammary glands, presumed to be the progenitors of the majority of cancers; or (ii) pregnancy suppressing cell division, which reduces the likelihood of the cells becoming frank carcinomas.

In conclusion, pregnancy reduced the percentages of ER- and/or PgR-positive cells and lowered the PCNA labeling index in the non-tumoral mammary glands of parous rats, resulting in suppression of the mammary carcinoma yield. Lactation had an additive effect on this reduction in mammary cancer. Since pregnancy appears to provide a physiological mechanism of mammary cancer prevention, it is important to elucidate the mechanism of the parity-related protection against mammary cancer; this information may lead to natural chemoprevention methods using pregnancy-related hormones.

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