Influence of the Estrous Cycle during Carcinogen Exposure on Nitrosomethylurea-induced Rat Mammary Carcinoma

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ABSTRACT

The influence of individual stages of the rat estrous cycle during exposure to l-nitroso-l-methylurea (NMU) on mammary tumor incidence, latency, number, and cytosol receptor dynamics for estrogen and progesterone was determined. Virgin female Buffalo rats were separated into three groups on Day 53 according to their vaginal smear patterns. NMU (5 mg/100 g body weight, i.v.) was administered in three monthly doses beginning at 53 to 55 days of age on diestrus, proestrus, or estrus between 9:00 and 11:00 a.m. Groups of rats had their second and third injections of NMU on the same day of the estrous cycle as their initial injection. All animals were killed during the morning of a diestrus day. Receptors for estrogen and progesterone were determined by a modified dextran-coated charcoal method and by sucrose density gradient analysis.

Mean latencies to first tumor appearance in diestrus, proestrus, and estrus groups were 104.4, 83.6, and 91.4 days, respectively (p < 0.05, diestrus versus estrus and proestrus) following the first NMU injection. The mean number of tumors per rat was significantly higher in rats injected on proestrus (4.5) or estrus (4.3) than on diestrus (2.0). Estradiol bound to receptor sedimented at 8 and 4 s and was suppressed by diethylstilbestrol and estradiol. Progesterone receptor migrated to 7.8 and 4 s regions. Estrogen receptor incidence (100%) and content (16.7 fmol/mg cytosol protein) was highest in rats injected on estrus. In the proestrus and estrus injected groups, estrogen receptor incidence was 95 and 63% and content was 10.2 and 11.2 fmol/mg protein, respectively. The affinity of estradiol for its receptor was not significantly altered in any group. Although there were no statistically significant differences in progesterone receptor incidence or affinity between groups, progesterone receptor content (74.6 fmol/mg cytosol protein) was significantly higher in tumors from rats injected on proestrus than on diestrus. These data suggest that the prevailing hormonal milieu of the estrous cycle during NMU exposure may be critically important to the subsequent biological behavior and steroid receptor status of carcinogen-induced rat mammary tumors.

INTRODUCTION

Estrogen and prolactin which induce mitosis in mammary epithelial cells have a synergistic and critical role in the induction of rat mammary tumors by chemical carcinogens (5, 8, 11). By promoting cellular replication, changes in circulating levels of estrogen and prolactin occurring at discrete intervals during the estrous cycle may alter mammary gland sensitivity to carcinogen exposure. As a result, mammary adenocarcinomas induced during certain phases of the estrous cycle may subsequently exhibit an altered latency, incidence, and hormonal responsiveness and account, at least in part, for the observed variation in tumor dynamics among rat mammary tumor models (12–15, 23–26, 31–33, 35).

Initial studies on the role of hormones in tumor induction by Young et al. (41) using DMBA3 administered p.o. suggested that the mean number of tumors per rat was significantly greater when DMBA was given on diestrus than when it was given at other stages of the estrous cycle. These results are compromised by the 16-hr delay in maximal DMBA mammary gland-DNA adduct formation (17) which would place peak DMBA binding and formation of active metabolites at the time of peak circulating estradiol levels. In contrast, later studies by Nagasawa et al. (27, 29) indicate that i.v. administration of DMBA at 6 p.m. on proestrus produced a greater number of tumors per rat, higher growth rate, and larger percentage of progressive mammary tumors than administration at 6 p.m. on diestrus. No difference was observed in tumor latency. Like those of Young et al. (41), these data may be compromised by the choice of carcinogen and the time of carcinogen administration. Prolactin levels are quite high on the evening of proestrus (30, 36) as is progesterone (30, 36) with estrogen having exerted its maximal effect hr previously (30, 36). The effect of any one hormone on tumor initiation in these studies is therefore difficult to establish. This suggests that i.v. administration of a direct-acting carcinogen, not requiring metabolic activation, with a very short half-life (<5 min) (38) might rapidly bind to maximally stimulated S-phase DNA (32, 33) and produce a decrease in tumor latency while increasing tumor number.

To investigate this hypothesis, we examined the relationship between the timing of NMU administration during various phases of the rat estrous cycle and mammary tumor latency, incidence, and presence of receptor for estrogen and progesterone.

MATERIALS AND METHODS

Tumor Induction and Assessment. One hundred twenty 40-day-old virgin female Buffalo (BUF/N) rats (Microbiological Associates, Bethesda, Md.) were housed in stainless steel cages, fed a commercial diet, and maintained in a 14-hr light-10-hr dark environment. Vaginal smears (0.9% NaCl solution lavage) were recorded daily throughout the study beginning at 43 days of age. On Days 53 to 55, animals were

1 The abbreviations used are: DMBA, dimethylbenz(a)anthracene; NMU, 1-nitroso-l-methylurea.

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weighed and separated into 3 groups according to stage of the estrous cycle. Twenty-seven rats (Group 1) were in proestrus (nucleated smear), 28 rats (Group 2) were in estrus (cornified smear), and 65 rats (Group 3) were in diestrus (leukocytic smear). All rats received NMU (Sigma Chemical Co., St. Louis, Mo.) (5 mg/100 g body weight; dissolved in 3% acetic acid:distilled water) i.v. (jugular) between 9 and 11 a.m. At 30-day intervals, each rat received 2 additional doses of NMU at the same stage of the estrous cycle as the initial dose. An additional 20 rats were given i.v. injections (10 on diestrus; 10 on proestrus) of 3% acetic acid in 0.9% NaCl solution to determine the effect of carcinogenic vehicle on estrous cyclicity. Those rats receiving NMU and not cycling regularly at the time of the second and third injection were not included in the study.

Mammary chains were palpated 3 times/week to determine initial tumor onset. Tumor latency was taken as the time (days) between the initial NMU injection and the appearance of a palpable tumor. Rats were sacrificed when morbidity appeared or at 150 days following the first NMU injection. Once established, tumor dimensions (measured with vernier calipers) were recorded weekly. Tumor volume was estimated according to the formula: length × width × depth × π/6.

Tissue and Histological Preparation. One hundred fifty days following NMU exposure (rat age, approximately 200 days), all animals were killed by CO₂ asphyxiation between 9 and 11 a.m. on a diestrus smear. Mammary tumors were dissected from surrounding structures, and representative specimens from each tumor were preserved in 10% buffered formalin. The remainder was immediately frozen over dry ice and maintained at −80°C prior to receptor assay. At autopsy, liver, spleen, and lung were examined grossly for metastases, and representative 5-μm paraffin sections of these tissues were routinely examined histologically for the presence of micrometastases.

Buffers and Reagents. Estrogen buffer (Buffer A) was prepared with 1.5 mM EDTA, 10 mM Tris-HCl, and 0.5 mM dithiothreitol (pH 7.4). Progesterone buffer (Buffer B) was prepared with 10 mM Tris-HCl, 1.5 mM EDTA, and 10 mM a-monothioglycerol containing 10% glycerol (v/v) (pH 7.4). Dextran-coated charcoal solutions for estrogen and progesterone receptor assays contained 0.25% charcoal and 0.0025% Dextran T-70 in Buffer A and 0.25% charcoal and 0.025% Dextran T-70 in Buffer B.

Radioiodinated 17β-[1,2,6,7-3H]estradiol and 17,21-[17α-methyl-3H]-dimethyl-19-nor-4,4-pregnadiene-3,20-dione (R5020) were obtained from New England Nuclear (Boston, Mass.). Radioinert steroids were purchased from Sigma and New England Nuclear.

Cytosol Preparation. Frozen tumor was pulverized in a Thermovac tissue pulverizer at liquid nitrogen temperature. All subsequent steps were performed at 4°C. Tumor samples (0.5 to 0.8 g) for estrogen receptor assays were diluted 1:10 (w/v) with Buffer A (1 to 2 mg/ml protein). Samples (1.0 g) for progesterone receptor assays were diluted 1:5 (w/v) with Buffer B (2 to 4 mg/ml protein). All specimens were homogenized in an ice bath with 3 bursts (10 sec) of a Polytron PT-10 homogenizer (Brinkman Instruments, Inc., Westbury, N.Y.) with inverting 30-sec cooling periods. The homogenate was centrifuged at 105,000 × g for 1 hr in a Beckman L8-70 Ultracentrifuge (Model Ti 70.1 rotor). An aliquot of the clear supernatant was retained for protein determination (21), and the remainder was assayed for estrogen and progesterone receptor.

Determination of Cytosol Hormone Receptor. The dextran-coated charcoal method used for assay of steroid receptor was similar to that described previously (10). Briefly, 200-μl aliquots of cytosol were added to 2 parallel series of 8 tubes each. One series contained 0.05 to 5 nM [3H]steroid plus 100-fold excess of unlabeled steroid to correct for nonspecific binding. Unlabeled hydrocortisone (10 nM) was added to all tubes in progesterone receptor assays to avoid nonspecific binding of R5020 to serum proteins and glucocorticoid receptor. Following incubation for 16 hr, unbound steroid was removed by exposure (20 min for estrogen receptor and 5 min for progesterone receptor) of each sample to the appropriate dextran-coated charcoal solution. The solution was centrifuged at 800 × g for 10 min, and the supernatant was decanted and counted in ACS cocktail (Amersham/Searle Corp., Arlington Heights, Ill.) in a Beckman LS 7500 spectrometer at an efficiency of 40%. Binding constants and total specific [3H]-steroid bound were calculated and analyzed according to Scatchard (34). A value of 3 fmol/mg cytosol protein was arbitrarily considered positive for either receptor. Continuous 10 to 30% sucrose gradients for estrogen and progesterone receptor determination by gradient centrifugation analysis were prepared in Buffers A and B, respectively. Cytosol was prepared (10 mg protein per ml) in a similar fashion to the dextran-coated charcoal assay and incubated with 5 nM [3H]estradiol or 20 nM [3H]R5020 for 4 hr. A parallel incubation was carried out with saturating concentrations (100-fold excess) of radioinert steroid. Free steroid was removed with charcoal, 200-μl aliquots were layered on 4.6-ml gradients, and tubes were centrifuged for 16 hr at 285,000 × g in a Beckman SW 50.1 rotor. Ten-drop fractions were collected and counted.

Statistics. Incidence of estrogen receptor- and progesterone receptor-positive tumors were compared by χ² while all means were analyzed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls a posteriori multiple comparison test (37).

RESULTS

Effect of NMU on the Estrous Cycle. Estrous cycles of sexually mature Buffalo rats are 4 (61%) or 5 (39%) days in length. NMU altered vaginal cyclicity in 28, 26, and 29% of rats treated by injection on diestrus, proestrus, and estrus, respectively. This was chiefly altering a 4-day cycle to a 5-day cycle for that cycle in which NMU was administered and did not depend on the estrous stage on which NMU was administered. The long-term abnormalities were of 3 types: (a) a constantly cornified vaginal epithelium for approximately 5 to 15 days followed by a leukocytic pattern for 5 to 15 days; (b) long (6 days) and highly irregular vaginal cyclicity; and (c) constantly leukocytic vaginal epithelium. Approximately 70% of those rats exhibiting abnormal estrous cycles following NMU injection returned to a regular 4- to 5-day pattern within one cycle of the first injection and continued to cycle regularly. The remaining animals injected exhibited abnormal vaginal smears throughout the remainder of the study. No animal demonstrating atypical cycles following the initial NMU injection was included in the data analysis. Two of 10 (20%) rats receiving the vehicle on diestrus switched from a 4-day cycle to a 5-day cycle for one cycle and then continued to cycle regularly.

Tumor Incidence, Latency, and Growth. Mammary tumors were present in 20 of 22 (91.6%) and 18 of 20 (90.0%) rats treated by injection on estrus and proestrus, while tumors developed in 39 of 47 (82.9%) rats treated by injection on diestrus (Table 1). Rats given injections on proestrus or estrus had a significantly greater number [4.5 ± 0.6 (S.E.) or 4.3 ± 0.8, respectively] of mammary tumors per rat than those given injections on diestrus (2.0 ± 0.16) (Table 1). Tumor latency was significantly longer in rats injected on diestrus when compared with those receiving NMU on either proestrus or estrus, although the time (150 days maximum allowed) for all tumors to appear was similar (Table 1). Fifty % of all rats injected during proestrus developed tumors within 83 days of initial NMU exposure, while 50% of the rats given injections during estrus and diestrus had developed tumors by Days 92 and 104, respectively (Chart 1).

Tumor growth rate was slower in those rats given injections on the morning of proestrus (doubling time, 10 days) than in those rats given injections on diestrus or estrus (doubling time,
Table 1

<table>
<thead>
<tr>
<th>Day of NMU exposure</th>
<th>First tumor appearance (days)</th>
<th>All tumors (days)</th>
<th>Incidence</th>
<th>No. of tumors/rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrus</td>
<td>104.4 ± 2.6 ± S.E.</td>
<td>115.0 ± 2.4</td>
<td>39/47 (82.9)c</td>
<td>2.0 ± 0.16</td>
</tr>
<tr>
<td>Proestrus</td>
<td>106.3 ± 6.9 ± S.E.</td>
<td>110.7 ± 7.2</td>
<td>20/22 (91.6)</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>Estrus</td>
<td>115.0 ± 2.4 ± S.E.</td>
<td>106.3 ± 6.9</td>
<td>18/20 (90.0)</td>
<td>4.5 ± 0.6</td>
</tr>
</tbody>
</table>

Mean ± S.E.

*p < 0.05, diestrus versus proestrus and estrus (ANOVA followed by Student-Newman-Keuls test).

Numbers in parentheses, percentage.

**p < 0.05, diestrus versus proestrus and estrus (ANOVA followed by Student-Newman-Keuls test).**

Not significant, diestrus versus proestrus and estrus.

Chart 1. Percentage of animals uninvolved with tumor with time following the first exposure to NMU. Δ, diestrus; ○, estrus; ●, proestrus.

7.5 days) (Chart 2).

Histology and Metastasis. Tumor specimens from all rats injected on diestrus, proestrus, and estrus that maintained regular cycles were morphologically indistinguishable. All tumors were adenocarcinomas with sheets and cords of cells many cell layers thick. Approximately 20% of the tumors from each group were papillary adenocarcinoma. Little or no fibrosis was present. No gross or microscopic metastases were observed in any tissue of any group.

Receptor Binding. The incidence and amount of assayable cytosol receptor for estrogen was highest in tumors from rats given injections of NMU on diestrus. Estrogen receptor content and incidence decreased significantly in tumors removed from rats given injections on proestrus and estrus, respectively (Table 2). Scatchard analysis revealed that estrogen binding to its receptor was consonant with a single class of high-affinity sites. Receptor affinity (expressed as Kd) of tumor cytosols from each group of rats was not significantly different. Sucrose density gradient analysis of tumor cytosols from rats injected on diestrus demonstrated migration to both 8 and 4 S regions, peak suppression by estradiol and diethylstilbestrol, and proteolysis by trypsin (20).

Although no statistically significant difference in incidence or affinity of receptor for progesterone was demonstrable among groups injected on diestrus, proestrus, or estrus, the incidence of rats bearing progesterone receptor-positive tumors rose from 44% on diestrus to 63% on estrus. Receptor content of tumor cytosols of rats injected on proestrus (74.6 fmol/mg protein) was significantly higher than those injected on diestrus (Table 2). Binding of progesterone to its receptor was also restricted to a single class of high-affinity sites. Sucrose density gradient analysis of tumor cytosols from rats injected on diestrus demonstrated progesterone receptor migration to 7.8 and 4 S regions, peak suppression by R5020, and proteolysis by trypsin (20).

DISCUSSION

These data demonstrate that the acute changes in endogenous hormonal milieu throughout the rat estrous cycle play a critical role in the initiation and development of carcinogen-induced mammary tumors. Although there was no difference in tumor incidence, tumor latency and number, as well as receptor dynamics, were all significantly and permanently influenced when 53- to 55-day-old virgin rats were given injections of NMU on different days of the estrous cycle. These observations confirm, in part, those of Nagasawa et al. (26, 27) but clearly show a significant decrease in tumor latency in proestrus and estrus injected rats. Again in contrast to Nagasawa et al. (27), who found that tumors from rats treated late on diestrus grow at a slower rate than those treated on proestrus, our data suggest that tumor growth rate was slower in those rats given injections on proestrus than in those given injections on diestrus and estrus. In spite of the shorter latency, those tumors arising in rats given injections on proestrus had a slower growth potential, perhaps reflecting an altered estrogen (or progesterone) responsiveness.

These significant alterations in tumor behavior are probably not due to a differential effect of NMU on hypothalamic-pituitary-o...
tary-ovarian function (as reflected by vaginal epithelial pattern) on a particular stage of the cycle, such as those reported for DMBA (19), since a similar incidence of estrous cycle perturbation was induced irrespective of the day on which NMU was administered. Less than 30% of the rats in each group exhibited abnormal cycles, and 70% of those returned to a regular 4- or 5-day cycle within one cycle of the first NMU injection. Although DMBA produces a long-term increase in proovulatory prolactin levels (19), the immediate effect of DMBA is to inhibit pituitary prolactin synthesis and release (16). It would appear, therefore, that differences in tumor dynamics may be due to early critical events in carcinogenesis rather than a generalized long-term effect on the hypothalamic-pituitary-ovarian axis.

Rat mammary carcinoma induced by aromatic hydrocarbons, such as DMBA, arise from the undifferentiated rapidly proliferating epithelial terminal end buds and terminal ducts present in the mammary gland of young virgin rats (32). The highest incidence of DMBA-induced mammary tumors arises in young (50- to 60-day-old) virgin rats when the number of terminal end buds and terminal ducts and the level of DNA synthesis is maximal (18). In addition to the maximal rate of DNA synthesis (1), the shorter length of the G\textsubscript{1} and S phases of the cell cycle in rats of this age (32) also increases mammary cell susceptibility to carcinogenesis.

DMBA and its active metabolites are not preferentially bound to newly replicated DNA (4), but the rate of DNA synthesis clearly influences both the amount of carcinogen bound (6) and subsequent tumor incidence (9). DMBA or any alkylating agent would therefore be expected to bind a greater extent to mammary gland DNA of young virgin rats, and does it, which correlates well with the higher level of estrogen-stimulated DNA synthesis in this age group (17). DMBA-DNA adduct formation peaks at 16 hr (17) following p.o. DMBA administration which approximates the range of the S phase of all undifferentiated mammary epithelial structures in young virgin rats (33). Cell culture studies suggest that maximal cell sensitivity to carcinogen-induced transformation occurs in late G\textsubscript{1} or during the S phase of the cell cycle (3, 22). It is during the S phase that the carcinogen apparently exerts its major action and transformation is permanently fixed (3, 22). In vitro, DMBA and other aromatic hydrocarbons require at least one cell division to take place before transformation occurs with at least 3 additional cell divisions necessary for full expression of the transformed state (3). These observations suggest that any synergistic action hormones would exert in initiating mammary tumor induction would probably reflect their prevailing physiological level during the period of maximal carcinogen exposure to mammary DNA. Clearly, the metabolic delay following p.o. and even i.v. administration of aromatic hydrocarbons would alter the time of peak exposure to DNA in relation to circulating hormone levels at the time of injection.

In sharp contrast to DMBA, the high chemical reactivity of NMU and short half-life in vivo (<5 min) make it highly unlikely that enzymatic metabolism is involved in the activation of the compound. In vivo and in vitro, NMU alkylation of O\textsuperscript{6} and N\textsuperscript{7} of guanine takes place by nonenzymatic heterolytic decompositions generating a methyl cation as the ultimate reactant in all tissues (38). Nitrosourea analogs of NMU produce DNA interstrand cross-links by rapidly adding a cation to O\textsuperscript{6} on guanine. Over a period of 6 hr, in the absence of free drug, they form an interstrand cross-link by the slow reaction of the bound cation with a nucleophilic site on the opposite DNA strand (7). Nitrosoureas also preferentially alkylate transcriptional chromatin (39). Nitrosourea-DNA adduct formation in HeLa cells stimulated by hydrocortisone pretreatment at concentrations which decrease DNA synthesis but stimulate transcription in HeLa cells in vitro leave RNA alkylation unaffected (39). These data are consistent with a steroid-induced relaxation of the supercoiled chromatin structure, resulting in increased transcription and increased accessibility of potential target sites for nitrosourea alkylation (39). A high level of circulating mitogenic hormones may induce a similar perturbation of mammary epithelial nuclear structure, thereby influencing the quantitative and qualitative interaction of NMU with sites involved in hormone-mediated cellular events. This could alter tumor initiation and the phenotypic expression of the subsequent mammary tumor.

Early in the 4- to 5-day rat estrus cycle, the 2- to 3-day-long metestrus-diestrus phase is characterized by low estrogen, progesterone, and prolactin levels. Estradiol begins to rise late on the second and early on the third day of the cycle (4-day cycle), peaks early on the morning of the following day (proestrus) 16 to 20 hr prior to ovulation (on estrus), and falls rapidly to basal levels during late proestrus (30, 36). Progesterone and prolactin begin to rise late on proestrus and fall to basal levels by the morning of estrus (30, 36).

Although it appears that prolactin has a direct mitogenic effect on normal and neoplastic rodent mammary tissue (40),...
there appears to be a lack of relationship between prolactin binding, the growth rate of DMBA-induced tumors, and circulating levels of prolactin in rats (28). Estrogen exerts its action directly on the mammary epithelial cell by initiation and shortening the length of the S phase of the cell cycle (1, 2, 6). We injected a direct-acting carcinogen, with a very short half-life, at a time of high and low circulating levels of estrogen and well in advance of the preovulatory rise in serum prolactin (30, 36). This suggests that the level of estradiol and/or prolactin at the time of NMU exposure altered mammary tissue sensitivity to carcinogen exposure. If NMU binding to DNA is a crucial step in carcinogenesis and occurs more readily in tissues undergoing rapid hormone-induced DNA synthesis, then one might anticipate an increase in tumor development during proestrus and estrus as demonstrated in the present studies. In addition, unoccupied estrogen receptor content of tumor cytosols would reflect the endocrine status of the animal at the time of NMU administration. The estrogen receptor content of mammary tumor cytosols from rats injected on proestrus was significantly lower than on diestrus. In contrast, the amount of progesterone receptor (induced by estrogen) was highest following a time of maximal estrogen secretion (30, 36). The receptor level differences cannot be explained by a variation in the endogenous hormonal milieu at the time of necropsy since all animals were killed early on diestrus (diestrus 2 in 5-day cycling rats). The low levels of estrogen receptor coupled with high levels of receptor for progesterone in tumors from proestrus injected rats may also help to explain the slower growth rate of these tumors.

Taken together, our observations suggest that the differences in the biological behavior of mammary tumors induced by NMU injection at various stages of the estrous cycle may result from NMU binding to segments of DNA which are either actively undergoing replication or transcription. Fluctuating levels of estrogen during the estrous cycle could predispose NMU binding to specific nucleotide segments, thereby influencing subsequent biological characteristics of the mammary epithelium and developing neoplasms.

One final point may be derived from the present study. As we have shown, the prevailing hormonal milieu during the estrous cycle can predispose mammary tissue to the development of carcinogen-induced tumors with divergent biological behavior. In the design of experiments to assess the influence of various hormonal manipulations on tumor dynamics and receptor content, we suggest that estrous cyclicity at the time of carcinogen administration should be assessed and standardized.

REFERENCES


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