Cellular Kinetics of Rat Mammary Gland Terminal End Bud Epithelium Exposed to \textit{N}-Methyl-\textit{N}-nitrosourea in Vivo^{1}

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ABSTRACT

Administration of the direct acting carcinogen \textit{N}-methyl-\textit{N}-nitrosourea (NMU) to 50–55-day-old virgin female rats on different days of the estrous cycle yields differential breast tumor biology (T. A. Ratko and C. W. Beattie, Cancer Res., 45: 3042–3047). One basis for these estrous cycle-dependent differences may be the duration of cell cycle stages of susceptible structures such as mammary terminal end buds or the quantity and duration of repair effected following adduct formation within these structures. The terminal end bud (TEB) epithelial cell cycle was characterized using pulse injections of \textit{H}\textit{^3}thymidine (0.5 mCi/g body weight). On estrus, TEB epithelial cell cycle was significantly shorter (15.5 h) than on proestrus (19.9 h) and diestrus (18.8 h). The shorter duration in TEB cell cycle on estrus was likely due to a shorter $T_{2G}$ (3–4 h) \textit{(P} \textless 0.05) since $T_2$ and $T_{2G}$ did not differ between estrous cycle days. When NMU was injected 1 h after \textit{H}\textit{^3}thymidine, the labeled mitotic wave within TEB of diestrus rats recovered ~2–3 h sooner than those given injections during proestrus \textit{(P} \textless 0.01), suggesting less initial damage or a slightly faster rate of DNA adduct repair. When \textit{H}\textit{^3}thymidine was injected 1–5 days after NMU, the percentage of labeled mitoses of rats given injections during diestrus and proestrus recovered to near normal 48 h after NMU, although the proportion of all cells labeled was still low compared to non-NMU-treated rats. The percentage of labeled mitoses and labeling of cells were normal 3 and 5 days after NMU. Rats receiving a carcinogenic but sublethal dose of NMU (5 mg/100 g body weight), followed by \textit{H}\textit{^3}thymidine injection within 1 min, had one-half the intensity of thymidine incorporation into the terminal end bud DNA of non-NMU-treated rats. Unscheduled DNA synthesis was not demonstrable within the first 48 h following injection of NMU. The results support and extend the finding that rat mammary epithelial cell carcinogenicity of NMU is estrous cycle dependent and appears to be correlated with a differential response in the cell cycle of TEB (shorter at estrus) or delayed recovery in response to NMU (proestrus versus diestrus).

INTRODUCTION

The biology of NMU\textsuperscript{4}–induced rat mammary carcinomas depends upon the stage of the estrous cycle at the time of carcinogen injection. Tumors induced by NMU injection on diestrus have a longer mean latency and lower incidence and number compared to tumors induced on proestrus or estrus (1–6). Serum levels of estradiol and prolactin are highest during the morning and afternoon of proestrus, respectively, and at a nadir in estrus, metestrus, and early diestrus, while progesterone is highest in late proestrus and again on metestrus (7, 8). Estrogen, in contrast to prolactin, increases human ductal DNA synthesis (9) and shortens the length of the 5 phase of the mammary epithelial cell cycle in mice (10, 11). Estrogen also appears to be essential in the induction and maintenance of NMU-induced tumors in the rat (12, 13). Similar studies using the longer acting 7,12-dimethylbenz(a)anthracene have also demonstrated an estrous cycle dependence in mammary tumor formation (14) and identified the rapidly proliferating cells of the relatively undifferentiated mammary TEB of 50–60-day-old virgin rats as a prime target for that carcinogen (15, 16). Since NMU is a direct acting carcinogen with a short half-life (10 min) \textit{(17) it, unlike 7,12-dimethylbenz(a)anthracene, can be used to dissect the potential influence of hormonal variations within the estrous cycle on tumor biology and determine whether these differences result from changes in duration of cell cycle parameters or in recovery of the cell cycle (3).\textsuperscript{5} In this regard, the $G_1$–$S$ border has been suggested as a critical stage for susceptibility to carcinogenicity (18–20). We therefore characterized the cell cycle in the relatively undifferentiated mammary TEB, of 55-day-old virgin rats, where cell proliferation is prominent, over each day of the estrous cycle and in response to NMU. Present results suggest that the recovery of the labeled mitotic wave is slower in mammary TEB of rats given injections of NMU during proestrus. Some of the results were presented previously (21).

MATERIALS AND METHODS

Animals. Two hundred thirty 38-day-old, virgin, virus-free female Sprague-Dawley rats (Harlan, Madison, WI) were housed in polycarbonate cages, fed a commercial diet and water \textit{ad libitum}, and maintained under a 12-h light/12-h dark lighting regimen. Estrous cycle stage was determined by daily vaginal saline lavage. At 50–54 days of age, rats exhibiting regular 4–5-day cycles were grouped according to stage and given injections of \textit{H}\textit{^3}thymidine or \textit{H}\textit{^3}thymidine under the following regimens. LMW. Rats were given i.p. injections of 0.5 \textit{mcg}/g body weight \textit{H}\textit{^3}thymidine (Amersham, Arlington Heights, IL; specific activity, 89 Ci/mmol) between 8 and 10 a.m. during proestrus (32 rats), estrus (37 rats), or diestrus/metestrus (36 rats) and killed over solid CO\textsubscript{2} at 2-h intervals over the next 24 h (Table 1, Experiment 1). The pelts with mammary glands attached were fixed by immersion in phosphate-buffered 10% formalin. After at least 24 h in fixative, mammary glands were dissected from the skin and stained in alum-carmine for 2–4 days, and samples of mammary epithelium with numerous TEB were processed through paraffin. Serial 5 \textmu m sections were coated with photographic emulsion by dipping in NTB-2 (Kodak, Rochester, NY) and stored in sealed black boxes with desiccant at 5°C for 2 weeks. The emulsion was developed (Kodak Dektol) and fixed (Kodak Fixer) and the sections were stained with hematoxylin and eosin. A single, thin, nonwinkled section was selected for each TEB, and labeled cells and mitoses (late prophase to late anaphase) as well as total population of cells in that section were counted. The threshold for a cell to be considered labeled was 4 grains/cell. Between 6 and 25 TEB involving an average of 3400 cells were counted for each rat (see Table 1, legend). The percentage of labeled mitotic cells was analyzed by a computer program

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\textsuperscript{4} The abbreviations used are: NMU, \textit{N}-methyl-\textit{N}-nitrosourea; TEB, terminal end bud; DE, diestrus; PE, proestrus; LI, labeling index; UDS, unscheduled DNA synthesis; LMW, labeled mitotic wave.

Table 1  Treatment regimens for the impact of NMU on mammary epithelium

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Notes</th>
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| 1          | [3H]Thd   | undisturbed LMW on proestrus, estrus, and diestrus during these stages of the reproductive cycle. In the first paradigm, injection of [3H]thymidine was followed after 1 h by injection of NMU which gave a measure of the effect of NMU on cells in $T_S$ or $T_G2$. The percentage of labeled mitoses from rats killed 2 or 4 h after injection of [3H]thymidine (1 and 3 h post-NMU) were not different from the control LMW curves (Fig. 2). Thus, cells contributing to the ascending limb of the

which assumes a $\gamma$ distribution on variability and establishes a maximum likelihood estimation by iteration, from which an estimate of cell cycle parameters ($T_C$, duration of cell cycle; $T_G1$, time between mitosis and DNA synthesis; $T_S$, duration DNA synthesis; $T_G2$, time between DNA synthesis and mitosis) was calculated (program provided courtesy of P. D. MacDonald (22). Since this is a mathematically determined best fit for the total body of data points, the approach limits variation from single time points.

NMU plus [3H]Thymidine. The cell cycle data provided a basis for examining the short term effect of NMU (Sigma Chemical Co., St. Louis, MO) on epithelial cells within TEB. Two groups of 50–54-day-old rats were given i.v. injections of 5 mg/100 g body weight NMU between 8 and 10 a.m. (3, 4). In the first group ($n = 75$) NMU was injected 1 h following an i.p. injection of [3H]thymidine (0.5 $\mu$Ci/g body weight). Rats were sacrificed at 2-h intervals over the next 29 h. Labeled mitoses were counted in TEB epithelium from rats given injections during proestrus (29 rats) and diestrus/metestrus (46 rats) (Table 1, Experiment 2). In the second group the order of injections was reversed (Table 1, Experiment 3), such that NMU injection was followed, up to 5 days later, by an i.p. injection of [3H]thymidine. Diestrous animals (51 rats) received [3H]thymidine immediately after the NMU, 1, 2, 3, 4, 6, 8, 10, and 18 h or 1, 2, 3, and 5 days later. Proestrus animals ($n = 22$) received [3H]thymidine at h 2, 4, 8, or 18 or 1, 2, 3, and 5 days later. Rats were sacrificed 4 h after injection of [3H]thymidine, a time at which a majority of mitoses are normally labeled in untreated rats, and tissues processed through paraffin as described above. The mean ± SE of percentage of labeled mitoses in TEB and percentage of cells mitotic for each interval sampled were analyzed by analysis of variance using a BMDP statistical software package (BMDP, Los Angeles, CA).

UDS. Unscheduled DNA synthesis was investigated in diestrous rats that received NMU (5 mg/100 g body weight) or vehicle, followed by 400 $\mu$Ci [3H]thymidine. Sustained blood levels of [3H]thymidine were established with four i.p. injections (4 × 100 $\mu$Ci) at 0.5-h intervals (23, 24) (Table 1, Experiment 4). The thymidine injections were started either within 1 min of i.v. NMU (NMU 1 min) or 48 h (NMU 48 h) after NMU and the rats were sacrificed 0.5 h after the last tritium injection. Mammary glands were dissected, fixed, stained, and sectioned as above and autoradiograms were exposed for 2, 4, or 8 weeks before development. Label intensity (grains/labeled cell) and proportion of cells lightly labeled (i.e., not in $T_S$ of cell cycle) with 2–6 or 7–11 grains were determined manually. Results were analyzed with Student’s $t$ test.

RESULTS

LMW. The labeled mitotic wave was calculated through a best fit analysis (Fig. 1). Estrus $T_C$ was calculated at 15.5 h (Fig. 1B; Table 2), with proestrus (Fig. 1A) and diestrus (Fig. 1C) at 19.9 and 18.8 h, respectively. TEB in estrous animals also had a shorter (~3–4 h) $T_G1$ phase than diestrous or proestrus rats (Table 2). Computer estimates of $T_S$ and $T_G2$ or their sum showed no significant difference between estrous cycle stages (Table 2).

NMU and LMW. Since NMU injection during diestrus can result in a significantly lower incidence of tumors with longer latency than injection during proestrus or estrus, we studied the response of mitotic cells to NMU under 2 different paradigms
control levels (Fig. 4), although the LI continued to be low expected 80–90%. Also, LI was about one-half that expected This suggests NMU was immediately cytotoxic and/or that received i.v. NMU and [3H]thymidine i.p. within 1 min follow
4 h after the [3H]thymidine injection, normally the apex of the TEB exposed to NMU were labeled with [3H]thymidine at se
synchronously (23).
not result in a large increase in mitoses, i.e., an overshoot phe
DNA synthesis was temporarily halted in a significant number of cells in 7's. Thus, of the mitoses present 4 h after the thymi-
percentage of labeled mitoses was 2 h post-[3H]thymidine (5+ h post-
percentage of labeled mitoses in TEB remained significantly below the
mitoses on the descending limb of the LMW in the two NMU-
percentage of labeled mitoses reached 65% through 12 h for DE
compared to controls the overall decline was delayed, 5 h in DE and 7.5 h in PE. The DE and PE data each fit a 2nd degree polynomial for h 4 through 16 postthymidine. Analysis of variance of regres-
sion coefficients over groups indicated a significant difference in DE versus PE (P < 0.01). At 16 h the percentage of labeled mitoses on the descending limb of the LMW in the two NMU-
injected groups were similar to that of the ascending limb of the second peak of the control LMW. NMU administered during proestrus and diestrus resulted in a significant (P < 0.001) drop in the incidence of mitotic cells (Fig. 3). Recovery of TEB did not result in a large increase in mitoses, i.e., an overshoot phe-
nomenon as might be expected if delayed cells were released synchronously (23).
NMU followed by [3H]thymidine. The next experiment looked at the recovery of the percentage of labeled mitoses in TEB by reversing the order of carcinogen and [3H]thymidine. TEB exposed to NMU were labeled with [3H]thymidine at selected intervals through 5 days (Fig. 4). All animals were killed 4 h after the [3H]thymidine injection, normally the apex of the LMW in control rats.
Control rats receiving [H]thymidine and killed 4 h later had 22.9 ± 1.8% of total cells labeled within TEB (LI), but rats that received i.v. NMU and [3H]thymidine in p. within 1 min following NMU injection had a LI of 16 ± 0.6% (P < 0.025, t test). This suggests NMU was immediately cytotoxic and/or that DNA synthesis was temporarily halted in a significant number of cells in TEB. Thus, of the mitoses present 4 h after the thymi-
dine injection, fewer were labeled. The maximum effect on the percentage of labeled mitoses was 2 h post-NMU when only 27% of cells were in TEB and available to incorporate [3H]thymidine and be counted as labeled mitoses 4 h later [i.e., 6 h after NMU (Fig. 4B)]. The 0-, 1-, 2-, and 4-h times were not repeated for PE (Fig. 4A). During the first 24 h post-NMU the percentage of labeled mitoses in TEB remained significantly below the expected 80–90%. Also, LI was about one-half that expected from controls (~15% versus 28%). By day 2 post NMU, the percentage of labeled mitoses in TEB had nearly recovered to control levels (Fig. 4), although the LI continued to be low (DE NMU, 16.1 ± 1.6% versus control, 24.0 ± 1.8, P < 0.05; and PE NMU, 13.2 ± 2.1% versus control, 27.9 ± 8.1, P < 0.1).
By day 3 the percentage of labeled mitoses in TEB had recovered to those values associated with an uninterrupted LMW (Fig. 4). Although additional early time points were taken in the experiment described in Fig. 4B, there was no significant difference in either the initial or overall pattern of recovery of the maximum percentage of labeled mitoses in TEB isolated from rats given injections of NMU during diestrus or proestrus.
UDS. A heavy and sustained exposure of TEB to [3H]thymidine was used to reveal any NMU-induced increase in overall

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>Metestrus/ diestrus (h)*</th>
<th>Proestrus (h)</th>
<th>Estrus (h)</th>
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<tbody>
<tr>
<td>Cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G₁</td>
<td>18.8</td>
<td>19.9</td>
<td>15.5</td>
</tr>
<tr>
<td>G₂</td>
<td>10.5 ± 0.6</td>
<td>12.1 ± 0.6</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>S</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>G₂</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean ± SE; computer analysis yields a maximum likelihood estimation of cycle parameters by repeated calculations for the best fit of the data set. Metestrus/ diestrus, 36 rats, 373 TEB, with 2,757 mitotic figures from 102,885 cells; proestrus, 32 rats, 418 TEB, with 3,023 mitotic figures from 131,380 cells; estrus, 37 rats, 439 TEB, with 3,402 mitotic figures from 128,721 cells.

Calculated TC₃.
receive an injection of carcinogen (2–5). Preneoplastic histological abnormalities induced by NMU are also estrous cycle dependent (6). Heavy damping of the LMW curves obviated the usefulness of looking for 3rd or 4th cell cycles. The 15–19 h duration of $T_C$ also meant that the cells within the mammary gland would be falling under a range of circulating hormonal changes on late diestrus and proestrus (7, 8) and therefore likely result in nonconstant kinetics (25–27). With this in mind, the TEB cell cycle duration reported by Russo et al. (15, 16) of 9.9 h in estrous rats calculated on a point to point basis is quite likely to be longer. Additional differences in present experimental design were to (a) limit the dose of $[^3H]$thymidine to one-third of previously reported amounts (15, 16) to avoid any possible radiation toxicity (28) and (b) hold the autoradiogram exposure constant at 2 weeks, instead of varying it from 2 to 4 weeks. While the first difference might enhance the viability and thus the cell cycle of the epithelium in TEB, the prolongation of exposure to 4 weeks might inappropriately enhance detection of minimally labeled cells.

With the LMW of TEB known for each day of the estrous cycle and available as a base line, the immediate effects of NMU on the TEB and its cells could be determined. A shorter $T_{G1}$ was

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**DISCUSSION**

The latency, incidence, number, and initial growth rate of NMU-induced rat mammary tumors differ significantly, depending upon the stage of the estrous cycle during which rats

**Fig. 3.** Percentage of TEB epithelium in mitosis. NMU was injected 1 h after $[^3H]$thymidine, and mitoses were counted irrespective of labeling. A, proestrus; $\triangle$, diestrus. $\bullet$, control; $\bullet\bullet\bullet\bullet$, NMU treated.

DNA synthesis during the first 48 h after NMU. After 2, 4, or 8 weeks of exposure of the autoradiograms, the grain density over cells given injections of $[^3H]$thymidine ($4 \times 100 \mu\text{Ci}$) starting within 1 min postexposure to NMU was one-half that of vehicle-treated controls (Table 3). In addition, untreated rats had significantly more TEB cells densely labeled than the NMU-treated rats (1 min) (Table 3) which further enhances the differences in labeling intensity between the two groups. Nuclei labeled 1 min and 48 h after exposure to NMU also had the same proportion of labeled cells in the categories of 2–6 and 7–11 grains/nucleus as found in controls (data not shown). The category 2–6 grains/nucleus was expected to increase in the response called UDS. NMU immediately and significantly, decreased the incorporation of $[^3H]$thymidine by cells in $T_S$. Also, new cells were not recruited into $T_S$ throughout the 2-h $[^3H]$-thymidine exposure, indicating a delay in the passage of cells from $T_{G1}$ into $T_S$.

**Fig. 4.** TEB recovery after NMU injection. NMU was injected at time zero, followed by $[^3H]$thymidine, at the times indicated. All rats were killed 4 h after the $[^3H]$thymidine (bars, SE) and are compared to the data for 4 h in the control LMW experiments (dots). Recovery pattern and full recovery by 3 days are similar in (A) PE and (B) DE rats.
observed for rats in estrus versus proestrus and diestrous, and a slightly faster $T_S$. Since $T_{G1}$ is also a time of production of proteins critical to entering $T_S$, a shorter $T_{G1}$ in estrus may mean that the expression of critical proteins and their mRNAs is more compressed in time influencing susceptibility to NMU. The shorter latency and higher tumor incidence and number in estrus/proestrus-treated rats (1–5) argue for this hypothesis. However, $T_{G1}$ in TEB epithelium of proestrus rats seems to be different from that of diestrous rats as seen in the delay of the descending side in the initial LMW when NMU was injected 1 h after thymidine. Thus, those cells exposed to NMU on PE 1 h after $[^3H]$thymidine take significantly longer to recover and proceed through mitosis. This correlates with a significantly higher amount of O6-methylguanine adduct retained in mammary epithelium of PE- versus DE-injected rats (4). The descending limb of the labeled mitoses curve is dependent upon uninterrupted recruitment into mitosis, i.e., cells in $T_{G1}$ at the time of $[^3H]$thymidine injection. Late $T_{G1}$ is correlated with production of proteins critical to entering S phase (30). Therefore, the shift in the descending limb supports the suggestion that sensitivity to NMU is maximal when cells are treated late in $T_{G1}$ on the $T_{G1}$-S border (18–20). The increased delay in the descending limb of the LMW in proestrus correlates with the higher incidence and shorter latency seen for mammary tumors and greater amount of mammary epithelial O6-methylguanine content following injection of NMU in proestrus.

To determine when mitosis recovers (~80–90% labeling) in TEB epithelium and when the total number of labeled cells rises to control levels, $[^3H]$thymidine was given post-NMU. The percentage of total TEB cells labeled had not fully recovered even at 48 h post carcinogen. Thus, in the initial stages of NMU-induced carcinogenesis the overall effect is one of cytotoxicity with a reduction in the rate of cell production. Dose-related delays in transit through $T_{G2}$ and $T_S$ have been reported following exposure of mouse fibroblasts or epidermis to NMU (20, 29). Perhaps the TEB cells that had to repair DNA before entering and completing mitosis were markedly delayed at each stage of the cell cycle. If, because of DNA damage, they continued to have delays in subsequent cell cycles, the total number of cells labeled might remain depressed. No difference was apparent between diestrous and proestrus in the recovery of the TEB epithelium in this latter experiment.

There was no increase in UDS under our paradigm of carcinogen followed by $[^3H]$thymidine administered over 2 h. The proportion of cells with 2–6 grains was not different between control and experimental groups. This differs from recent observations with esophageal epithelium (31) and tracheal rings in vitro (32), where there was direct exposure of cells to carcinogen rather than circulating levels. Direct exposure could be expected to result in very high, localized concentrations of carcinogen at the epithelium, and substantially more DNA damage. The lack of UDS detection correlates with the maximal effect of NMU occurring at late $T_{G1}$. Thus, as cells repaired the late $T_{G1}$ damage, they might proceed to $T_S$ and become heavily labeled which would not be recognized as UDS. The decrease in rate of $[^3H]$thymidine incorporation immediately after NMU was similar to findings after direct application of NMU to mouse epidermis (29), although $[^3H]$thymidine incorporation into TEB cells in the present study recovered essentially to control levels by 48 h. The O6-methylguanine adduct has been implicated in carcinogenesis and will be at least partially repaired by the alkyl guanine-DNA alkyltransferase (4, 5, 33) present in rat mammary epithelium and not be detected as $[^3H]$thymidine substitution. Other DNA repair mechanisms such as excision repair could include $[^3H]$thymidine incorporation.

In short, basic TEB cell cycle characteristics may contribute to the estrus/proestrus stage sensitivity to NMU (1–6). The different cell cycle responses to NMU by TEB in proestrus versus diestrous also indicate such a contribution. Overall, data lend support to the hypothesis that TEB epithelium is more susceptible to damage at the $G_1$-$S$ border than at the late S and $G_2$ phases (18–20).

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