Effect of Tamoxifen on Preneoplastic Cell Proliferation in N-Nitroso-N-methylurea-induced Mammary Carcinogenesis

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ABSTRACT

N-Nitroso-N-methylurea (NMU) is an effective carcinogen for the induction of mammary carcinoma in the rat. Tamoxifen (TAM), used as a chemopreventive agent to reduce tumor incidence, has been well studied using this model. We have utilized the rat mammary carcinoma model to assess the effect of TAM on preneoplastic changes. Fifty-day-old virgin female Sprague-Dawley rats were randomized by weight and divided into the following five groups: Group 1, normal controls (n = 24); Group 2, TAM (n = 20); Group 3, NMU-short term (n = 24); Group 4, NMU-short term + TAM (n = 26); and Group 5, NMU-long term (n = 23). Seven weeks after the exposure to NMU, rats in Groups 1, 2, 3, and 4 were given injections of [3H]thymidine and sacrificed 4 h later for autoradiographic determination of thymidine labeling index (TLI). The rats from Group 5 were observed for 30 weeks after NMU exposure to confirm mammary tumor development. TLI in both terminal ducts and terminal end buds was modulated by treatment with TAM. Carcinogen administration induced higher TLI relative to the normal controls (18.3 ± 1.8% (SD) versus 15.5 ± 2.1%, P < 0.001) in terminal end buds. The effect of carcinogen on TLI was also apparent in the terminal ducts (15.8 ± 1.1% versus 9.5 ± 1.1%, P < 0.001). TAM administration was able to suppress both constitutive and NMU-induced TLI increases in terminal end buds (15.5 ± 2.1% versus 2.8 ± 1.1% and 18.3 ± 1.8% versus 6.8 ± 1.4%, respectively, P < 0.001). Similar effects were observed in terminal ducts. In addition to its antiproliferative effect on nontransformed mammary tissue, TAM was effective in suppressing NMU-induced mammary tumor incidence and frequency. NMU-induced hyperproliferation is an intermediate stage in NMU carcinogenesis in the rat and is suppressed by TAM. Mammary epithelial hyperproliferation may provide a useful quantitative intermediate end point to evaluate chemopreventive efficacy.

INTRODUCTION

The American Cancer Society estimates that 175,000 women will be diagnosed with breast cancer in 1991 which will prove fatal for over 44,000 women and that 1 in 9 women face a lifetime risk of this disease (1). Improved survival in breast cancer patients is best achieved by early detection. However, discovery of early or "minimal" breast cancer with its attendant high cure rates has only recently led to attempts to describe a preneoplastic state in breast tissue. Studies of the preneoplastic state and the effect of potential preventive agents may assist in the development of strategies for primary prevention.

Preneoplasia may be most simply defined as any morphologically identifiable lesion at risk for malignant transformation. In the rodent model, such specific lesions as ductal and/or lobular hyperplasia have been identified as preneoplastic lesions. Upon transplantation, these lesions produce overt carcinomas at the transplant site in recipient animals (2, 3). These hyperplastic lesions therefore are at higher risk than normal tissue for developing carcinoma and, as such, may provide a morphological intermediate marker for cancer risk. Recently, hyperproliferation of the target tissue in response to a carcinogenic insult has been identified as a cellular intermediate biomarker for cancer risk (4). Specific genetic, endocrine, and pathological intermediate biomarkers for cancer risk in humans are not always easily identifiable. It is important to identify and validate alternative intermediate biomarkers that may permit study of strategies designed to improve survival. Down-regulation of these specific and sensitive biomarkers of cancer risk may provide useful quantitative end points to evaluate the potential efficacy of chemopreventive agents.

Chemopreventive agents are many and varied, both synthetic and natural, operating through several modes of action. Some agents are truly preventive; others modify existing precancerous lesions (5). There is evidence supporting the hypothesis that the development of breast cancer is dependent on estrogenic stimuli. Tamoxifen, a relatively selective antiestrogen, has been shown to decrease the incidence of new primary breast cancers in the contralateral breast of early stage breast cancer patients (6). Because of its low toxicity TAM is an attractive option for endocrine intervention. Large randomized, prospective trials are currently under way in England and proposed in the United States to evaluate TAM as a primary preventive agent (7). Apart from studies demonstrating its therapeutic antitumor effects in humans and animals, TAM has demonstrable chemopreventive effects in experimental animal mammary carcinoma (8). Tamoxifen reduced the number of tumors as well as delay tumor appearance in the 7,12-dimethylbenz(a)anthracene model of rat mammary carcinogenesis (9), and similar observations have been made in the NMU model (10). TAM has an additional advantage that it can act as an effective chemopreventive agent when administered after tumor initiation at a point where early subclinical cancer is present (11).

It is well established that the development of atypical hyperplasia in ducts, terminal ductules, and terminal end buds in the rat mammary carcinoma model is associated with increased cell proliferative activity (12, 13). In this model, TAM administration has been noted to suppress appearance of mammary tumors but little is known about the antiestrogenic influence on the early events of tumorigenic transformation such as hyperproliferation. The aim of the present study was to examine the effect of TAM on the postinitiation phase of NMU-induced carcinogenesis that precede the emergence of adenocarcinomas.

MATERIALS AND METHODS

Animals. One hundred seventeen virgin female Sprague-Dawley rats were obtained at 42 days of age (Charles River Breeding Laboratories, Wilmington, MA). Animals were housed 2/cage in a room illuminated on a 12L:12D cycle. Food and water were available ad libitum. All animals were handled in accordance with the guidelines established by the Animal Care Committee of the Cornell University Medical College.

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for 12 h each day and maintained at a temperature of 25 ± 1°C. Throughout the experiment, all animals had free access to Purina laboratory chow (Ralston-Purina, St. Louis, MO) and drinking water.

Chemicals. Crystalline NMU was purchased from Ash-Stevens, Inc. (Detroit, MI) and was dissolved in 0.85% NaCl solution acidified to pH 5.0 with acetic acid. The concentration was then adjusted to 10 mg/ml.

TAM citrate (Stuart Pharmaceuticals, Wilmington, DE) was administered in a 0.2-ml peanut oil vehicle. Four days after NMU administration, treatment with TAM was begun and continued for 38 days. TAM was given via s.c. injection, 5 times/week, at a 100 µg/day dose in the TAM and NMU-S + TAM groups. All other animals received a s.c. injection of peanut oil on the same schedule.

Experimental Design. At 55 days of age, the rats were randomized by weight and divided into the following five groups: Group 1 (normal control, n = 24) received a single i.v. injection of 0.85% saline (pH 5.0); Group 2 (TAM, n = 20) received 100 µg TAM s.c. five times/week; Group 3 (NMU-S, n = 24) received a single injection of NMU (50 mg/kg body weight) via the tail vein and was observed for 49 days after carcinogen exposure; Group 4 (NMU-S + TAM, n = 26) received a single injection of NMU via the tail vein and s.c. injections of TAM five times/week. This group was also observed for 49 days; Group 5 (NMU-L, n = 23) received a single injection of NMU and was observed for 204 days after carcinogen exposure.

Seven weeks after carcinogen exposure, Groups 1–4 received an i.p. injection of 1 µCi/g body weight of tritiated thymidine (specific activity, 25 Ci/mmol; Amersham-Searle, Arlington Heights, IL). The animals were weighed and killed 4 h after injection. Group 5 was observed weekly for tumor development, animals were killed when moribund. They were observed for at least 204 days after NMU treatment.

Tissue Preparation. The lumbar mammary glands 4, 5, and 6 in the normal control, TAM, NMU-S, and NMU-S + TAM groups were removed, fixed in 10% buffered formalin, and processed for histology and autoradiography. Mammary tumors were coded as to location, and representative tissues were taken and prepared for histological study. Tissue sections were stained with hematoxylin-eosin and classified histopathologically (14). Only histologically confirmed mammary carcinomas were used in the data analysis.

Autoradiography. Deparaffinized 5-µm-thick sections were coated with NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY) and stored for 2–4 weeks in lightproof boxes at 4°C. The slides were developed in Kodak D-19 developer and fixed in Kodak acid fixer, and the autoradiographs were stained with hematoxylin and eosin (15).

Microscopic Analysis. The TLI was defined as the ratio of the number of labeled nuclei (=5 grains/nucleus) to the total number of cells scored. The epithelium of TEBs and TDs in both longitudinal sections and cross-sections was evaluated. The TLI was expressed as the number of labeled nuclei per 100 epithelial cells. The data were derived from the total number of evaluable TD and/or TEB from representative sections of the mammary glands from each treatment group. The mean & SD were calculated for each group.

Statistical Analysis. Significance for mean TLI was tested by analysis of variance. Differences in tumor incidence were determined using the Kruskal-Wallis and Hollander analysis with the Wolfe multiple comparison test.

RESULTS

Effect of TAM on Postinitiational Events. Histologically, the mammary epithelial component exhibited large ducts ending in TEB; this arrangement shows a similarity with a previously published schematic representation (16). Autoradiography permitted observation of the various anatomic components of the mammary gland in the process of cell proliferation. Random observation of Groups 1–4 revealed consistent, sporadic appearance of [3H]thymidine-labeled cells in TD as well as in TEB. The [3H]thymidine label was predominantly restricted to the epithelial component of the mammary gland, indicating significant participation of this component in the cell proliferative activity of the organ.

The data presented in Table 1 show the proliferative activity observed in the mammary epithelium of the TD and TEB. Forty-nine days after NMU exposure there was a significant increase in TLI in both TD and TEB (P < 0.001). This increase was modifiable by treatment with TAM since the NMU-S + TAM group revealed a significant decrease in TLI in both TD and TEB (P < 0.001). The antiproliferative effect of TAM on the noninvolved mammary epithelium was evident from the TLI values of the TAM group which were lower than those from the normal control group (P < 0.001). The TLI data were analyzed by the anatomic location and the extent of labeling. The results presented in Table 2 show that the majority of TEB exhibited less than a 5% TLI and there were no statistically significant differences between the groups. However, when TEB exhibiting >5% TLI were analyzed, NMU-S group showed increased number of labeled TEB relative to that in the control group (P < 0.001). The NMU-induced increase was modified in the presence of TAM as evidenced by a substantial decrease (P < 0.001) in the number of labeled TEB in NMU-S + TAM group in comparison with those in NMU-S group. The antiproliferative effect of TAM on normal epithelium unexposed to carcinogen is also evident by a substantial decrease (P < 0.001) in the number of labeled TEB in TAM group relative to that observed in the control group.

To exclude the possibility that changes in cell proliferative activity have occurred due to toxicity of NMU-S and/or TAM administration, survival and changes in total body weights of the rats in all the five groups were determined. In rats that were observed for a duration of 49 days, all the treatment groups showed 100% survival. Rats in TAM, NMU-S, and NMU-S + TAM groups, however, exhibited a suppression in weight gain ranging from about 20 to 50 g relative to the normal control group (P < 0.001).

Effect of TAM on NMU-induced Tumorigenesis. The incidence of mammary tumors in control group and TAM group

Table 2 Frequency and distribution of [3H]thymidine labeling in mammary TEB

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total</th>
<th>Mean</th>
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<th>&lt;5%</th>
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</thead>
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<td>7.8*</td>
<td>53.1</td>
</tr>
<tr>
<td>4</td>
<td>1131</td>
<td>43.5</td>
<td>2.3*</td>
<td>41.2</td>
</tr>
</tbody>
</table>

* P < 0.001.
was unable to induce mammary tumors in noninitiated mammary glands. In the NMU-S group, 6 animals of 24 examined (25%) had mammary tumors with a frequency of 0.36 tumor/animal. In NMU-S + TAM group only 1 animal of 26 examined showed tumor. Thus, in this group the tumor incidence and frequency were 3.8% and 0.04, respectively. A comparison of NMU-S and NMU-S + TAM groups demonstrates that administration of TAM to carcinogen-initiated epithelium can effectively suppress tumor development.

The NMU-L group, which was observed for 204 days after NMU exposure, had a 70% survival. However, all the surviving animals in this group had mammary cancer, with a frequency of 2.04 tumors/rat.

**DISCUSSION**

Chemical carcinogen-induced mammary carcinoma in the rat has been the most extensively used animal model for human breast cancer (17). Similar to human breast cancer, most carcinogen-induced rodent tumors are multicentric (18) and estrogen responsive (18), and the tumor incidences and latent periods are modifiable by hyperalimentation with dietary fat, antiestrogens, or retinoids (17, 19–22). Most of the studies have utilized the appearance of overt carcinoma as the only end point (19). Mammary tumorigenesis is a multiphasic process, and the presence of overt carcinoma is the last phase of disease progression prior to metastasis. Thus, suppression of tumor growth by antiestrogens or retinoids may be a reflection of their ability to prevent growth of cancer (21). These studies therefore provide evidence for chemotherapeutic rather than chemopreventive efficacy of dietary interventions or hyperalimentation of retinoids and TAM. Intermediate biomarkers of cancer risk capable of predicting tumorigenic transformation could also function as important intermediate end points to evaluate the chemopreventive efficacy. In this report, we have utilized NMU-induced alteration in cell proliferative activity that precedes the appearance of mammary carcinoma as the intermediate biomarker and have examined the ability of TAM to counteract the short term effects of NMU administration.

Alteration in the cell proliferative activity in response to a carcinogenic insult or to the administration of tumor-promoting diets has been noted to occur in the animal models (12, 13, 22, 23). These experimental manipulations induce enhanced cell proliferation in the target tissue well before the appearance of mammary cancer. Induced hyperproliferation has been considered as an intermediate biomarker of cancer risk (23, 24). Our results on an altered [3H]thymidine labeling index in response to NMU and/or TAM administration demonstrate that NMU treatment induces increased [3H]thymidine uptake in the cells of TD as well as of TEB. Since these two sites constitute a major epithelial component of the mammary gland, it is conceivable that proliferative changes induced by NMU on the transformation-sensitive target tissue may be relevant in tumorigenic transformation. In this context, it is interesting to note that antiproliferative effects of TAM are detectable not only in normal but also in carcinogen-initiated mammary epithelium. These observations suggest that preventive effect of TAM may in part be due to its antiproliferative activity on the mammary epithelial component. Classical mammotrophic hormones such as estrogen, progesterone, and prolactin are known to profoundly influence cell proliferation, and cytodifferentiation as well as tumor development in the mammary gland (8–10, 13, 16, 17). It is conceivable that in the present in vivo study TAM-mediated alteration in TLI of mammary epithelial component may in part be due to antiestrogenic activity of TAM and/or to indirect alterations in circulating levels of other hormones such as progesterone and prolactin.

Terminal end buds have been considered as the presumptive target epithelial component for tumorigenic transformation (2, 3, 12, 13). We therefore examined the extent of TLI in this component of the mammary gland and the alteration in TLI induced by TAM, NMU-S, and NMU-S + TAM. These experiments revealed a wide variation in TLI. The number of TEB with <5% TLI was greater than that of TEB showing >5% TLI. TAM administration was clearly effective in inhibiting constitutive as well as carcinogen-induced cell proliferation. The data presented in Tables 1 and 2 taken together indicate that the initial response of the mammary epithelium to NMU exposure is manifested as hyperproliferation in the cells of TD and TEB, and TAM can effectively counteract this effect of the carcinogen. Since NMU-induced hyperproliferation of non-transformed epithelium is suppressed by TAM, well before the evidence of tumorigenic transformation, this may constitute an intermediate biomarker for carcinogenic response as well as indicate the efficacy of a potential chemopreventive agent.

From our results it is clear that NMU administration results in increased TLI (hyperproliferation) in the mammary epithelial component. Suppression of hyperproliferation by TAM suggests that for the biological effect of TAM on preinitiated cell, altered hyperproliferation may be a relevant end point. It is noteworthy that TAM also suppresses TLI in noninitiated epithelium. In this context, altered proliferation may serve as an appropriate cellular end point for the effect of TAM.

It is conceivable that systemic factors may indirectly influence the effect of TAM on the mammary tissue in vivo. In the experiment conducted to examine the systemic toxicity of the various treatments, total body weights were determined. TAM administration induced about 16–20% body weight reduction relative to the control as evidenced by a suppression of the weight gain by about 20–50 g. This systemic effect did not overtly affect normal morphogenesis and development of the mammary epithelial component. Despite a decrease in total number of TEB, the mean numbers of TEB in the two TAM groups were comparable. Consistent with these observations, TAM selectively inhibited the genesis of preneoplastic mammary lesions in mice without affecting the overall growth of the epithelial component in the target tissue (25).

The validity of alteration in cell proliferative activity as an intermediate biomarker for mammmary cancer risk is also evident from the experiment examining the effects of NMU and TAM on tumor incidence. Group 3 after exposure to NMU showed elevated [3H]TLI and increased tumor incidence as well as increased tumor frequency. In Group 4, suppression in [3H]TLI paralleled a reduction in tumor incidence as well as in tumor frequency. As expected, a comparison between Group 2 and Group 5 revealed a higher tumor yield in the latter group confirming the validity of model.

In conclusion, this study provides evidence that carcinogen-induced alteration in cell proliferative activity constitutes an intermediate marker for breast cancer risk in the rat. This end point should be further studied to assess its utility in evaluating the chemopreventive efficacy of naturally occurring as well as synthetic compounds.
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