Maternal Exposure to Tamoxifen during Pregnancy Increases Carcinogen-induced Mammary Tumorigenesis among Female Rat Offspring

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ABSTRACT
Tamoxifen is under investigation as a potential chemopreventive agent in women of child-bearing age who are at an increased risk to develop breast cancer. However, because tamoxifen may act as an estrogen in the fetus and high fetal estrogen activity, in turn, may increase subsequent breast cancer risk, we wanted to determine the effects of a maternal exposure to tamoxifen during pregnancy on offspring's susceptibility to mammary tumorigenesis. Pregnant rats were injected s.c. with 20 μg of tamoxifen or oil vehicle daily during days 15 and 20 of gestation. In utero exposure to tamoxifen caused abnormalities in the development and function of the reproductive track, including a delayed puberty onset and changes in uterine wet weights. The tamoxifen-exposed offspring, treated with 7,12-dimethylbenz[a]anthracene (DMBA) at the age of 45 days, developed an increased incidence of mammary tumors. In week 18 after DMBA administration, 50% of the vehicle-controls had developed mammary tumors, whereas tumor incidence in the tamoxifen group was 95%. In addition, a significantly higher number of tumors in the tamoxifen-exposed group kept growing (rather than stopped growing or regressed) than in the control group. Furthermore, histopathological examination revealed that the mammary tumors in the tamoxifen offspring were less differentiated and exhibited a more aggressive phenotype, compared with the tumors growing in the controls. These results suggest that a maternal exposure to tamoxifen during pregnancy acts as an estrogen in the fetal mammary gland and increases the susceptibility to breast cancer among female offspring.

INTRODUCTION
The partial estrogen agonist, tamoxifen, successfully reduces the recurrence of breast cancer (1) and is widely used as adjuvant breast cancer therapy after surgery. It also reduces the incidence of breast cancer among high-risk women who have never been diagnosed with the disease (2). Because tamoxifen is being studied as a chemopreventive agent, its use may expand to include more women of child-bearing age. Tamoxifen therapy is not recommended during pregnancy, mainly because it may cause fetal abnormalities (3). For example, animal studies have linked a maternal exposure to tamoxifen during pregnancy with abnormalities in the reproductive tracts of the offspring (4). Some of these abnormalities are similar to those caused by fetal exposure to the synthetic estrogen diethylstilbestrol (5); others are specific to tamoxifen.

Estrogenic manipulations during fetal life through a pregnant mother alter breast cancer risk among daughters. Indicators of high pregnancy estrogen levels, including high birth weight (6, 7), birth jaundice (8), and being a twin (9, 10), increase daughters’ breast cancer risk. Indicators of reduced pregnancy estrogen levels, including hypertension/preeclampsia, are associated with reduced breast cancer risk (8). We studied, using a well-established animal model, whether a maternal exposure to tamoxifen during pregnancy affects carcinogen-induced mammary tumorigenesis among female offspring. Previous results obtained using this model indicate that in utero exposure to estradiol through a pregnant mother increases the offspring’s mammary tumor incidence (11). Our goal was to determine whether tamoxifen acts as an estrogen in the fetal mammary gland and increases breast cancer risk.

MATERIALS AND METHODS
Animals. Female Sprague Dawley rats, purchased from Charles River Breeding Laboratories, were obtained on day 10 of gestation. The animals were housed singly, in standard rat plexiglass cages, at a constant temperature (20–22°C) and humidity (60–65%), under a 12-h light-dark cycle (lights on for 6.00 h). Two days after the offspring were born, the males were killed, and the females were cross-fostered to lactating dams that had been exposed to the same treatment during pregnancy as the pups it was to nurse. Ten to twelve female pups were housed with each lactating dam. The female offspring were weaned on postnatal day 22, and, thereafter, were housed in groups of three to five animals. Our experimental protocol was reviewed and approved by the Georgetown University Animal Care and Use Committee.

Tamoxifen Exposure. The dams were treated daily with either 20 μg of tamoxifen ([Z]-1-[dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) from Sigma Chemical Co. (St. Louis, MO) or with vehicle (2% DMSO in peanut oil), administered as s.c. injections in a volume of 0.05 ml, between days 15 and 20 of pregnancy.

Inducing and Monitoring Mammary Tumorigenesis. Mammary tumors were induced by the administration of 10 mg of DMBA3 (Sigma) to 45-day-old offspring. The DMBA dose was suboptimal to produce sufficient tumorigenesis to allow evaluations of both reductions and increases in the end points of tumorigenicity. The carcinogen was dissolved in peanut oil and administered by oral gavage in a volume of 1 ml. The vehicle group contained 24 offspring; and the tamoxifen group contained 22 offspring.

The animals were checked for mammary tumors once a week. The end points for data analysis were (a) latency to tumor appearance; (b) the number of animals with tumors (tumor incidence); (c) the number of tumors per animal (tumor multiplicity); and (d) the proportion of tumors that were proliferating. A tumor was designated as proliferating if it increased regularly in size. Tumor sizes were measured by recording the tumor diameters with a caliper and determining the length of the longest axis and the width perpendicular to the longest axis. The animals were killed when detectable tumor burden approximated 10% of total body weight. The surviving animals, as well as animals that did not seem to develop mammary tumors, were killed at week 18 after carcinogen administration.

Thirteen DMBA-induced mammary tumors from tamoxifen or vehicle offspring were evaluated with a blind method for histopathological changes. The tumors were subjectively graded into well-, moderately, or poorly differentiated adenocarcinomas. The invasive features were also recorded when observed. Immunohistochemical staining for PCNA was carried out to further assess the proliferative potential of the tumors.

RESULTS

Effects on Early Development. Tamoxifen did not have any adverse effects on pregnancy, including body weight gain in the mothers and offspring, when compared with the vehicle treatment (Table 1). However, several parameters of physical and reproductive system development were significantly affected by the maternal exposure to tamoxifen (Table 1). Eyelid opening was delayed in the offspring of mothers exposed to tamoxifen during pregnancy (two-tailed Student’s t test = 3.75; df, 89; P < 0.001). Uterine wet weights on day 21, determined by weighing the tissues after they had been kept on 1% PPS for approximately 3 h, were significantly lighter in the tamoxifen than in the vehicle offspring (t = 2.18; df, 52; P < 0.034). Our previous studies indicate that rats exposed to estradiol in utero do not differ from appropriate controls in terms of uterine wet weights (11) and exhibit advanced eye-lid opening4 and vaginal opening (11). Thus, the effects on physical development and reproductive system of a maternal treatment with tamoxifen differed from those seen in rats whose mothers were exposed to estradiol during pregnancy.

Effects on Mammary Tumorigenesis. Mammary tumors began to appear 6 weeks after carcinogen administration, as also reported by other investigators (12). The animals were followed for 18 weeks after DMBA, at which time the animals were killed. The results indicate that in utero exposure to 20 μg of tamoxifen per day, through a pregnant mother, during days 15 and 20 of gestation, significantly increased the incidence of DMBA-induced mammary tumors when compared with the vehicle group (log-rank test: z-value = 2.73, P < 0.006; Fig. 1). On week 18, 50% of the vehicle-controls and 95% of the tamoxifen offspring had developed mammary tumors (x2 = 7.42; df, 1; P < 0.01). The latency of tumor appearance and tumor multiplicity were similar in the tamoxifen and vehicle groups (Table 2). Thus, the rats exposed to tamoxifen in utero were at a higher risk to develop mammary tumors than the controls, but there were no apparent differences in the tumor latency.

Our (13) and other investigators’ (14) earlier data have shown that the majority of mammary tumors induced by DMBA given at the age of 45–50 days are adenocarcinomas, regardless whether they proliferate or not. Proliferative DMBA tumors may have a more aggressive phenotype than nonproliferative tumors. In the present study, there were more tumors that consistently grew between measurements in the offspring of tamoxifen mothers than in the offspring of vehicle mothers (x2 = 7.42; df, 1; P < 0.01).

Histopathological examination confirmed that the tumors in the tamoxifen offspring were more aggressive. All (100%) of

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3 The abbreviations used are: DMBA, 7,12-dimethylbenz[a]anthracene; PCNA, proliferating cell nuclear antigen; TEB, terminal end bud; df, degree(s) of freedom.

4 E. Cho and L. Hilakivi-Clarke, unpublished data.

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Table 1  Pregnancy- and nursing-related variables in female rats exposed to vehicle or to 20 μg of tamoxifen between days 15 and 20 of gestation

<table>
<thead>
<tr>
<th>Maternal treatment</th>
<th>Vehicle</th>
<th>Tamoxifen</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pregnant animals</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>No. of litters</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>% successful pregnancies</td>
<td>78</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Length of pregnancy (days)</td>
<td>21.4 ± 0.2</td>
<td>21.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>No. of pups/litter</td>
<td>10.0 ± 2.2</td>
<td>10.8 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>% females per litter</td>
<td>56.0 ± 10.5</td>
<td>45.5 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>Pup weight (g) on day 2</td>
<td>8.1 ± 0.4</td>
<td>7.8 ± 0.3</td>
<td></td>
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<tr>
<td>Pup weight (g) on day 7</td>
<td>13.7 ± 0.6</td>
<td>13.7 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Pup weight (g) on day 14</td>
<td>27.9 ± 3.3</td>
<td>27.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Pup weight (g) on day 21</td>
<td>46.0 ± 4.1</td>
<td>48.2 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Age at eyelid opening (days)</td>
<td>9.4 ± 0.1</td>
<td>9.9 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uterine wet weights on day 21 (mg)</td>
<td>476 ± 44</td>
<td>328 ± 7</td>
<td>&lt;0.016</td>
</tr>
<tr>
<td>Age at vaginal opening (day)</td>
<td>28.1 ± 0.2</td>
<td>28.8 ± 0.3</td>
<td>&lt;0.034</td>
</tr>
</tbody>
</table>
Maternal tamoxifen exposure increases the number of proliferating tumors

Table 2

<table>
<thead>
<tr>
<th>Tumor latency (weeks)</th>
<th>Tumor multiplicity (tumors per rat)</th>
<th>% of proliferating tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (<em>n = 16/12</em>)</td>
<td>10.8 ± 0.7</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Tamoxifen (<em>n = 30/21</em>)</td>
<td>12.2 ± 0.7</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> *n*, number of tumors/number of animals with tumors.
<sup>b</sup> Significantly higher than the vehicle group, *P* < 0.01.

Maternal tamoxifen exposure increases the number of proliferating tumors

Data represent the mean ± SE of latency to tumor appearance, tumor multiplicity, and proportion of proliferating tumors in DMBA-treated rats that were exposed to vehicle or to 20 µg of tamoxifen in utero.

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The present study indicated that tamoxifen, when administered through a pregnant mother at the dose of 20 µg/day for 5 days during the last week of gestation, significantly increased the incidence of DMBA-initiated mammary tumors. Additionally, this treatment facilitated the development of a less differentiated, more aggressive (invasive) tumor phenotype with higher proliferating potential, as shown by the larger number of PCNA-labeled cells. Although these findings are in agreement with our unpublished data showing a strong correlation between highly proliferative DMBA-induced mammary adenocarcinomas and low differentiation grade, they are in sharp contrast to the data obtained in adult animals that indicates that the treatment of adult rats with tamoxifen after a carcinogen administration reduces both tumor proliferation and progression (15). The discrepancy may be due to the fact that in adult animals, tamoxifen acts by binding to estrogen receptor and inhibiting its activation. During fetal life, however, tamoxifen may activate estrogen receptor when bound to it and, thus, act as a receptor agonist rather than an antagonist. Results obtained in female reproductive systems after in utero exposures support this interpretation (4, 13, 16). Furthermore, when adult, virgin rats are exposed to tamoxifen, it is likely that tumorigenesis results from a direct interaction of tamoxifen with estrogen receptor, whereas in the case of fetal exposure, the effect of tamoxifen may be a consequence of the fetal imprinting of the mammary gland.

A possible mechanism mediating the agonistic effect of tamoxifen exposure in utero on the mammary gland is a tamoxifen-induced increase in the number of TEBs in the mammary glands. Our earlier data have shown that maternal exposure to a high estrogenic environment increases the number of TEBs in the developing mammary gland first and then the incidence of carcinogen-induced mammary tumors (11, 17). In utero exposure to tamoxifen also increases the presence of TEBs (13). TEBs contain highly proliferative epithelial cells (i.e., stem cells) and are the sites that give rise to carcinogen-induced rodent mammary tumors (12). The equivalent structures in the human breast are the terminal ductule lobular units (18), which also are thought to give rise to the majority of human breast cancers (19). The present results, which show an increase in mammary tumorigenesis, and the earlier results, which showed more TEBs (13) by an in utero tamoxifen exposure, further support the hypothesis that the higher the number of TEBs at the time that the carcinogen is administered, the higher the risk of breast cancer.

Previous studies indicated that fetal tamoxifen exposure has several adverse effects on the reproductive system. Mice that are treated with approximately 100 µg of tamoxifen daily during pregnancy give birth to offspring that exhibit progressive proliferative hyperplasia of the oviduct, uterine lesions, and ovarian tumors (4). Perinatal tamoxifen treatment (range, 1–50
pregnant, should avoid using tamoxifen.

If true for humans, our data suggest that women who become pregnant while taking tamoxifen may increase their daughters’ breast cancer risk. Thus, our findings add to the concerns raised by other investigators as to the safety of tamoxifen during pregnancy seems to slow postnatal development in the offspring. This delayed development is not caused by a slowing in general growth, inasmuch as body weights were similar in the tamoxifen and control offspring. If true for humans, our data suggest that women who become pregnant while taking tamoxifen may increase their daughters’ breast cancer risk. Thus, our findings add to the concerns raised by other investigators as to the safety of tamoxifen during pregnancy and underline the importance of the directive that pregnant women, or women who may become pregnant, should avoid using tamoxifen.

REFERENCES
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