Tamoxifen Inhibition of Estrogen Receptor-α–Negative Mouse Mammary Tumorigenesis

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Abstract
Tamoxifen reduces the relative risk of breast cancer developing from specific premalignant lesions. Many breast cancers that arise after tamoxifen treatment are estrogen receptor-α (ER-α)–negative, although premalignant lesions such as atypical ductal hyperplasia are highly ER-α–positive. The p53 null mouse mammary epithelial transplant model is characterized by ER-α–positive premalignant lesions that give rise to both ER-α–positive and ER-α–negative tumors. Given this progression from ER-α–positive to ER-α–negative lesions, we tested the ability of tamoxifen to block or delay mammary tumorigenesis in several versions of this model. In groups 1 and 2, p53 null normal mammary epithelial transplants were maintained in virgin mice. In groups 3 to 5, the p53 null and mammary transplants were maintained in mice continuously exposed to high levels of progesterone. In groups 6 and 7, transplants of the premalignant outgrowth line PNA8a were maintained in virgin mice. Tamoxifen blocked estrogen signaling in these mice as evidenced by decreases in progesterone-induced lateral branching and epithelial proliferation in the mammary epithelium. Tamoxifen did not alter the elevated levels of progesterone in the blood while significantly reducing the circulating level of prolactin. Tamoxifen reduced tumor incidence in p53 null normal mammary epithelial transplants maintained in virgin mice from 55% to 5% and in progesterone-stimulated mice from 81% to 21%. The majority of the resultant tumors were ER-α–negative. Tamoxifen also significantly delayed tumorigenesis in the ER-α–positive high premalignant line PNA8a from 100% to 75%. These results show that tamoxifen delays the emergence of ER-α–negative tumors if given early in premalignant progression. (Cancer Res 2005; 65(8): 3493-6)

Introduction
Breast cancer is the major cancer among women in the United States in terms of noncutaneous cancer incidence and the second leading cause of cancer deaths (1). Approximately 40% to 50% of primary breast cancers are either estrogen receptor-α (ER-α)–negative or ER-α–positive but estrogen unresponsive (2). Thus, a substantial proportion of breast cancers are not responsive to hormonal therapy. ER-α–positive cancers have a slightly better prognosis; however, the major benefit of the ER-α–positive phenotype is the ability to treat with antiestrogen therapy (3). Whereas the selective estrogen receptor modifer (SERM) tamoxifen has been used for many years to treat ER-α–positive breast cancer, in recent years, SERMs such as tamoxifen and raloxifene, have been used to prevent the development of breast cancer (4–6). Indeed, tamoxifen is now approved for reducing breast cancer risk in high-risk women. However, several large prevention trials have shown that antiestrogens such as tamoxifen and raloxifene reduced the incidence of only ER-α–positive breast cancer; these drugs did not reduce the incidence of ER-α–negative breast cancer. ER-α–negative breast cancers arise more commonly in premenopausal women (7), in African American women (8), and in BRCA1 carriers (9). For these women, effective preventive options are urgently needed.

The presence of an ER-α–negative cancer does not necessarily mean that the development of the cancer is estrogen independent. The literature on human premalignant lesions suggests that many prevailing lesions are ER-α–positive (10). Suspected premalignant lesions, such as usual ductal hyperplasia, atypical ductal hyperplasia (ADH), and ductal carcinoma in situ are often ER-α–positive. Additionally, the percentage of mammary epithelial cells positive for ER-α within a given premalignant lesion is often much higher than that found for the normal terminal ductular lobular unit (10). Recent studies show that tamoxifen reduces the relative risk of cancer developing from specific premalignant lesions (e.g., ADH) by 87% (4). Interestingly, the vast majority of the cancers that do arise after tamoxifen treatment are ER-α–negative. The origin of ER-α–negative breast cancers is still uncertain. It is equally plausible that such cancers arise from cells that are inherently ER-α–negative or from cells in which ER-α expression has been down-regulated during premalignant or malignant progression. Thus, a critical question remaining is whether antiestrogen therapy, if given early in the development of breast cancer, will be able to reduce the incidence of both ER-α–positive and ER-α–negative breast cancer.

In the past 5 years, there have been several new transgenic and knockout mouse models of breast cancer. These models are based on genes known to be deregulated in human breast cancer and include p53 (11–13), neu (14–17), BRCA1 (18), BRCA2 (19, 20), p53 and Rb (21, 22), and c-myc (23). The vast majority of the breast cancers arising in these transgenic mice, as in the traditional mouse mammary tumor virus (MMTV) models such as the C3H mice (24), are ER-α–negative. In some of these models, the premalignant progression is hormone responsive as evidenced by delayed tumorigenesis in ovariectomized or tamoxifen-treated mice (15, 22–26). The magnitude of the effect of hormone manipulation varies depending on the model with a modest delay seen in the SV40 Large Tag model (22) and a more significant delay seen in the neu (15) and p53 null models (27).

The p53 null mammary epithelium model has been characterized at the biological and genetic level in detail (11, 12, 27). The tumors are aneuploid, metastatic, and primarily ER-α–negative (about 20% of the primary tumors are ER-α–positive). However, the p53 null normal mammary epithelium is ER-α–positive and absolutely dependent on ovarian steroid hormones for growth, functional

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differeniation, and tumorigenesis (12). Thus, this model is a well-characterized transgenic model that develops both ER-α–positive and ER-α–negative mammary tumors. Hormonal stimulation by estrogen and/or progesterone or prolactin/progesterone markedly enhances tumorigenesis (11, 27). Blocking estrogen signaling by either ovarioectomy or blocking progesterone signaling by knocking out the progesterone receptor greatly reduces tumorigenic capability of the p53 null mammary epithelium (27). Given the marked hormone dependence of the p53 null normal mammary epithelium and the strong response to prolonged hormone stimulation, we tested the ability of the SERM, tamoxifen, to block or delay tumorigenesis in this model. In this brief communication, we report the ability of continuous tamoxifen exposure to markedly delay the development of ER-α–negative tumors arising in p53 null mammary cells.

Materials and Methods

BALB/c mice (both p53 wt and p53 null) were bred and maintained in a closed conventional mouse colony at the Baylor College of Medicine with food and water provided ad libitum and the room temperature set at 70°F. The animal facility is Association for Assessment and Accreditation of Laboratory Animal Care accredited. The basic experimental protocol was as published in ref. (11). Mammary duct samples from 8- to 10-week-old BALB/c p53 null or from the premalignant outgrowth line PN8α (12) were transplanted into the cleared mammary fat pads of 3-week-old BALB/c p53 wt mice. Mammary epithelial cells of the PN8α line express ER-α in 10% of the cells. The transplanted ducts grow and fill the mammary fat pads in 6 to 8 weeks. The morphologic and differentiation deficiencies of the mammary epithelium associated with p53 loss are limited to a delay in the first stage of involution (11, 28, 29). The mice were divided into seven groups. Groups 1 to 5 received p53 null normal mammary duct from 8-week-old female BALB/c p53 null mice. Groups 1, 2, and 3 were maintained as virgin mice. Groups 4 and 5 were implanted s.c. with a silastic tubing containing 20 mg progesterone at 5 weeks of age. The continuous increased circulating level of progesterone results in differentiation of the mammary epithelium and sustained elevation in cell proliferation (30). The silastic tubing was replaced at 6-week intervals. Groups 2 and 5 received a tamoxifen pellet (5 mg) implanted s.c. in the upper back at 11 weeks of age. The pellets were replaced at 3-month intervals. Groups 6 and 7 were implanted with pieces of PN8α premalignant outgrowth line. Group 7 received a tamoxifen pellet (5 mg) at 11 weeks of age. There were 20 to 33 transplants per group for the tumor study. Mice were palpated weekly for tumors over the study period. The tumor incidences were evaluated statistically by Fisher's exact test (groups 1-5) or log-rank test (groups 6-7). In addition, several fat pads containing the transplants were collected from groups 1 to 5 at 14 weeks post-transplant (3 weeks of tamoxifen treatment) and examined for histology, ER-α and progesterone receptor immunohistochemistry, and proliferative activity [bromodeoxyuridine (BrdUrd) labeling] as described in ref. (12).

We do not have information on the pharaoconomics of release of the drug in the pellets. However, we do note that the 5 mg dose is significantly lower than the 28 mg dose used by Jordan et al. (26). In those studies, the 28 mg dose resulted in blood levels of tamoxifen of 24 to 4 ng/mL over a 6-month period.

The circulating concentrations of progesterone and prolactin were measured using RIA as previously described (31). Blood was collected from groups 3 to 5 at 32 weeks post-transplant. There were five to six samples per group. ANOVA and Fisher's protected-least-significant-difference test or nonparametric analysis of paired groups using Wilcoxon signed rank test were applied for comparing the hormonal concentrations in serum of the different animal groups. In all cases, P ≤ 0.05 was considered significant.

Results

As expected, the presence of elevated circulating levels of progesterone with no added tamoxifen induced an increase in lateral branching and an increase in proliferative activity by 5-fold compared with untreated, nonstimulated mammary epithelium (BrdUrd LI = 30:500 versus 5:6:500, respectively). Tamoxifen treatment of the hormone stimulated epithelium partially reduced proliferation of the progesterone treated cells from 30:500 to 16:500. These results show that tamoxifen blocked estrogen signaling in the p53 null mammary epithelium in the predicted manner. Tamoxifen had absolutely no effect on the elevated blood levels of progesterone caused by the progesterone released from the silastic tubing (Table 1). However, the serum concentration of prolactin was significantly decreased in the animals that received the steroid treatment and were also implanted with tamoxifen pellet compared with untreated animals and animals receiving progesterone only.

The tumorigenic response of the p53-null mammary epithelium in groups 1 and 2 is shown in Fig. 1A. Tamoxifen decreased the spontaneous tumor incidence 91% from 55% (11 of 20) in the untreated mice to 5% (1 of 20) in the treated mice at 56 weeks post-transplantation. Tumorigenesis in groups 3 to 5 are shown in Fig. 1B. The presence of progesterone increased the tumorigenic response (P ≤ 0.05) compared with that of untreated epithelium (21 of 33, 82% versus 8 of 33, 24%), at 45 weeks post-transplantation. The addition of tamoxifen completely eliminated the enhanced tumorigenic response induced by the chronic progesterone stimulation (tamoxifen = 7 of 33, 21%); thus, this group effectively behaved like the untreated group. In groups 3 to 5, 4 of 19 tumors (21%) were strongly positive for ER-α expression as detected by immunohistochemistry. ER-α–positive tumors appeared in each of the three groups. The effect of tamoxifen on tumorigenesis in the ER-α–positive high premalignant line PN8α was slight but significant by log-rank test (Fig. 1C). The overall tumor incidence was decreased by 25% (100% in the untreated mice to 75% in the tamoxifen-treated mice). The decreased incidence occurred primarily late after transplantation (weeks 26-32). The resultant tumors were ER-α–negative.

Discussion

The results presented here show that tamoxifen suppresses both ER-positive and ER-negative mammary tumor development in the p53 null mammary gland model. These results also imply that the development of ER-negative breast cancer is dependent on the ER at some point in its genesis. Our results seem at first analysis to be at odds with extensive clinical data from cancer prevention trials showing that SERMs (such as tamoxifen or raloxifene) effectively suppress ER-positive breast tumorigenesis but do not suppress ER-negative tumor development (4, 5, 32). However, our preclinical model and the human clinical trials differ in one important aspect. In the studies reported here, we treated mice with mammary glands responsive to progesterone and prolactin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Progesterone (ng/mL), mean ± SE</th>
<th>Prolactin (ng/mL), mean ± SE</th>
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<tbody>
<tr>
<td>Sham</td>
<td>1.1 ± 0.3*</td>
<td>16.4 ± 6.1</td>
</tr>
<tr>
<td>P4</td>
<td>11.3 ± 1.3</td>
<td>17.8 ± 9.7</td>
</tr>
<tr>
<td>P4 and TAM</td>
<td>11.1 ± 1.3</td>
<td>12 ± 0.2†</td>
</tr>
</tbody>
</table>

*P < 0.05, significantly lower than P4 and P4 and Tam.  †P < 0.05, significantly lower than P4 and Sham.
that treatment in early adulthood may suppress both ER-positive and ER-negative tumor development, whereas treatment later will only suppress ER-positive tumor development. This is a testable hypothesis using our p53 null mammary gland model. Such experiments are now ongoing.

One reason for the effectiveness of tamoxifen is the marked hormone dependence of premalignant progression of the p53 null mammary epithelium. Previous results have shown that the absence of p53 does not disrupt normal hormone dependence and responsiveness of the mammary epithelium (11, 12, 27, 29). Furthermore, hormone responsiveness is maintained into late stages of premalignant development (12). Traditional and other transgenic mouse models for breast cancer have been tested for their tamoxifen responsiveness. In virgin C3H mice, tamoxifen given continuously from 3 months of age significantly blocked mammary tumorigenesis and was more effective than ovariectomy (26). The superior effectiveness of tamoxifen in this mouse model was likely due to blocking estrogen signaling at the ER whereas the absence of estrogen by removing the ovaries is eventually compensated by adrenal hyperplasia, which provides a new source of estrogen (33, 34). In the SV40 Large T antigen model, tamoxifen did not cause a significant delay in tumorigenesis (22). In the neu model, tamoxifen causes a significant delay in tumorigenesis if tamoxifen is started at 12 weeks of age but not if started at 24 weeks of age (15).

The response of PN8a to tamoxifen is reminiscent of the response of the neu mammary gland. Tamoxifen is effective blocking early stages of premalignant progression but relatively ineffective on premalignant cells late in progression. Another interpretation, which is not mutually exclusive to that offered above, is that mammary cells undergoing rapid genetic change, are not susceptible to tamoxifen-induced prevention. The estrogen receptor-α status of the tumors in neu mice has recently been reported as negative (35); however, the ER-α status of the premalignant lesions and the hormone dependency of the premalignant progression in the neu mice have not been reported.

The important issue of the origin of ER-α-negative tumors is not addressed in this study. The ER-α-negative tumors might evolve from an ER-α-positive precursor cell or from an ER-α-negative cell that is critically dependent during its premalignant stage on surrounding ER-α-positive cells. However, the current results do support the concept that the development of ER-α-negative cancers is dependent at some point on estrogen signaling.

The effect of tamoxifen on mammary tumorigenesis may not be exclusively exerted at the mammary tissue level. The results reported herein, as shown earlier (36), show that tamoxifen treatment sharply reduces the circulating concentration of prolactin in the progesterone-treated mice. Overexpression of prolactin in the mouse mammary gland results in a high incidence of mammary tumorigenesis and these tumors can be either ER-α-positive or ER-α-negative (37). Deletion of prolactin in prolactin knock out mice results in attenuated development of the mammary duct (38). However, the effect of prolactin in increasing tertiary duct branching and in tumorigenesis is mediated through increasing progesterone levels (39) and in our study, tamoxifen did not alter progesterone levels.

Tamoxifen treatment also attenuates GH release in both rats (40) and humans (41) and decreases insulin-like growth factor-I (IGF-I) concentration in serum of rats (42) and humans (41). Thus, the inhibition of GH/IGF-I axis could be significant in light of recent evidence supporting a fundamental role of this axis in mammary tumorigenesis (43, 44).

The p53-null mammary epithelium model used in this study seems an appropriate model as it mimics several of the important
characteristics found in human breast cancer development. First, most normal cells and cells in the premalignant lesions express ER-α and progression of these lesions to invasive breast cancer is estrogen and progesterone responsive. Second, the premalignant phenotype is genetically unstable as is true for the comparable human lesions. Third, hormone manipulation, by SERM or ovariotomy markedly delays the onset of tumorigenesis; yet, the tumors that emerge are ER-α-negative. This unique model of both ER-α-positive and ER-α-negative mammary tumorigenesis offers an opportunity to study molecular characteristics of these two subtypes of breast cancer in the same animal model. For example, this model may be particularly useful to address the effect of duration and timing of tamoxifen exposure on ER-α-positive and ER-α-negative mammary tumorigenesis as these determinants have recently been shown to be effective in developing chemoprevention strategies for experimental breast cancer (45, 46). It would be important to determine if a limited duration of tamoxifen exposure (3 months) in our p53 null model is effective in inducing a long lasting prevention of mammary cancer as occurs in the traditional MMTV C3H model (26). In addition, because the mammary tumors that arise in these mice are both ER-α-positive and ER-α-negative, this model would be appropriate to test the combination of antiestrogens and other cancer preventive agents.

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References

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