Prevention of age-related spontaneous mammary tumors in outbred rats by late ovariectomy

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Abstract

Background: Breast cancer prevention trials have shown that the antiestrogen tamoxifen inhibits development of estrogen receptor (ER)-positive tumors. In Sprague–Dawley rats, removal of ovarian function in young animals can reduce the incidence of spontaneous age-dependent mammary tumors. However, it is not known whether removal of ovaries late in life, before middle age onset, can still prevent mammary tumor development. Methods: In this study we used Hsd:Sprague–Dawley® SD® (Hsd) rats to determine the effect of late ovariectomy on mammary tumor development. Intact, sham-ovariectomized and ovariectomized rats were followed until 110 weeks of age, or over their life span. In some experiments, palpable tumors were surgically removed upon presentation. Results: Removal of ovaries before middle age onset (~5–7 months) inhibited development of spontaneous mammary tumors by 95%. Only one mammary tumor was observed in 19 late ovariectomized animals while 47 total tumors developed in 42 non-ovariectomized animals. Tumor incidence was reduced from 73.8 to 5.3% (relative risk = 0.05, 95% CI = 0.0072–0.354). The frequency of mammary carcinomas in non-ovariectomized virgin female rats was one in eight rats. Spontaneous rat carcinomas expressed ER and other biomarkers, such as cyclin D1. When palpable tumors were removed by surgical excision, tumor multiplicity increased from 0.76 to 1.61 tumors per rat. Surprisingly, ovariectomy increased the 110-week survival rate and maximum life span of Hsd rats. Conclusion: Late ovariectomy prevents spontaneous mammary tumor development in Hsd rats. This animal model may be useful for evaluating novel interventions in breast cancer prevention.

Keywords: Cancer prevention; Breast cancer; Aging; Ovarian function; Estrogen receptor; Cyclin D1; Tumor multiplicity; Animal model; Overall survival; Life span

1. Introduction

One in eight women develop breast cancer over their life span. A key risk factor in breast cancer is age, although not all types of breast cancer are affected similarly by aging. Most breast cancers in older women (70–80%) express estrogen receptor (ER) alpha, whereas only 50% of breast tumors in younger women are ER-positive [1]. This age-dependent increase in the incidence of ER-positive, but not ER-negative, breast tumors is well documented [1]. ER-positive tumors are estrogen-dependent and can respond to hormonal treatments, such as treatment with the selective estrogen receptor modulators tamoxifen or with aromatase inhibitors, that block estrogen synthesis. Moreover, breast cancer prevention trials have shown that the selective estrogen receptor modulators, such as tamoxifen and raloxifene, can efficiently reduce ER-positive breast tumors in high-risk women [2–7].

To investigate new prevention strategies for age-related breast cancer, an animal model that develops spontaneous age-dependent ER-positive breast cancer is desirable. The rat has been useful in studies of estrogen-responsive breast tumors due to the presence of ER in most rat mammary tumors while absent in most mouse mammary tumors [8]. In particular, Sprague–Dawley rats have been used successfully to study the role of hormones in breast cancer development and the protective effects of hormonal therapies in preventing carcinoma formation after carcinogen treatment [8–11]. Like humans, the incidence of spontaneous mammary tumors in Sprague–Dawley rats is age-dependent [12–14].
Tumor incidence in outbred Sprague–Dawley rats depends on several variables including type of diet, amount of caloric intake, environment and source of the Sprague–Dawley stock [10,15–17]. To our knowledge, the first study showing the requirement of intact ovarian function in spontaneous formation of mammary tumors in Sprague–Dawley rats was published over 40 years ago [13] using rats ovariectomized at a young age (≤3 months). A more recent study, confirmed the observation that early ovariectomy (≤3 months) reduces spontaneous mammary tumor formation in aging Sprague–Dawley rats [18]. However, as intervention strategies in women are typically started later in life we sought to determine the effect of late ovariectomy in spontaneous mammary tumor development using Sprague–Dawley rats. For this study, we used the Harlan (Hsd:Sprague–Dawley® SD®) stock (Hsd). This stock was selected because Hsd rats have lower body weight with aging and a lower incidence of pituitary tumors than rats from the Charles River (Crl:CD® (SD) Br) stock, commonly used for toxicological studies [19,20]. We observed that late ovariectomy efficiently reduced the development of spontaneous age-related mammary tumors in Hsd rats, without decreasing overall survival.

2. Materials and methods

2.1. Animal treatments

Two different experimental designs (protocols) were used in this study. The goal of the first protocol was to compare the cumulative incidence of spontaneous mammary tumors in virgin female Sprague–Dawley rats in the presence of ovarian function or its absence since middle age onset. Two independent experiments were conducted following this protocol. The first experiment had a total of 23 animals (12 intact, 5 sham-ovariectomized and 6 ovariectomized) and animals were observed until 110 weeks of age. The second experiment had a total of 14 animals (7 sham-ovariectomized and 7 ovariectomized). In this second experiment from the first protocol animals were followed through their life span. The goal of the second protocol was to compare tumor multiplicity and overall life span in the presence or absence of ovarian function (earlier or later in life). In this second protocol, mammary tumors or other palpable tumors exceeding 2.0 cm in diameter were either euthanized (first protocol, 110-week and life span experiment) or underwent surgery for tumor removal (second protocol, overall survival experiment). If surgery was successful, the rat was housed alone for a period of 2 or 3 weeks for observation and then placed back in its original cage. In all experiments, animals were euthanized if they were deemed ill, off-feed or moribund according to standard guidelines [21]. The mammary glands of the euthanized animals were analyzed for the presence of mammary tumors. To assess tumor multiplicity, mammary tumors were considered distinct only when observed in different mammary glands. Upon death of the animal, several tissues such as uterus, ovaries and pituitary were removed for further analysis when possible and ovariectomy was confirmed at this stage. Complete necropsy was done only if the cause of death of the animal could not be readily determined.

2.2. Histology and immunohistochemical analyses

Mammary tumors were immersion-fixed in 10% neutral buffered formalin for 24 h and paraffin embedded. Serial 6 μm paraffin sections were stained with hematoxylin and eosin for histopathological diagnosis. Mammary epithelial tumors were classified as fibroadenomas, adenomas or adenocarcinomas as appropriate. For immunohistochemistry, slides were baked for 1 h at 60 °C and deparaffinized. Sections were then subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (Vector Laboratories, Burlingame, CA) for 10 min and allowed to cool for 10 min. Endogenous peroxidase was quenched by incubating slides for 10 min in 3% H2O2 in dH2O. Nonspecific binding was blocked by incubating sections for 30 min in 5% bovine serum albumin/0.5% Tween. In addition, to block endogenous biotin present in mammary tissue, sections were then incubated in the Avidin portion of ABC kit (Vector Laboratories) for 30 min. After rinsing in phosphate buffer saline, sections were incubated with the specific primary antibody diluted in 5% bovine serum albumin, overnight at 4 °C. Mammary carcinomas were immunostained for ER (MC-20, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (Ab-3, NeoMarkers, Fremont, CA) and progesterone.
receptor (PR) (Santa Cruz). The following day, the slides
were rinsed in phosphate buffer saline and exposed to the
appropriate Vector Elite ABC kit as per manufacturer’s
instructions. Sections were counterstained with hematoxylin
(Sigma–Aldrich, St. Louis, MO).

2.3. Statistical analyses

For analysis of mammary tumor-free survival curves, the
Kaplan–Meier method was used with log-rank tests for
group differences. To compare the numbers of animals with
tumors or 110-week survival, the Fisher’s exact test was
used. For comparison of tumor multiplicity, the unpaired
two-tail t-test was used. For all statistical analyses the
PRISM software was used (GraphPad Inc., San Diego, CA).

3. Results

3.1. Age and hormone-dependent mammary tumor
incidence on Hsd rats

To determine the effect of late ovariectomy on
spontaneous mammary tumor development we first carried
out a 110-week study with female Hsd rats, in the presence
or absence of ovarian function. Removal of ovaries was
performed before rats entered into middle age (i.e., <7
months old). This time was chosen because age-dependent
alterations in estrous cycles are observed in Sprague–
Dawley rats during middle age (7–9 months old) [22]. Fig. 1
shows the incidence of mammary tumors as percent of
mammary tumor-free survival [10] in normal (intact) virgin
rats compared to ovariectomized or sham-ovariectomized
virgin rats. Intact or sham-ovariectomized Hsd rats showed
age-dependent increases of mammary tumors. Late ovar-
iectomy significantly decreased mammary tumor formation
when compared to sham-ovariectomized animals (Fig. 1 ,
\( P = 0.0124 \)). Tumor multiplicity (number of tumors per rat)
was 0.76 for non-ovariectomized Hsd rats while 0 for
ovariectomized animals. While most age-dependent mam-
mary tumors were benign, we observed two mammary
 carcinomas in 17 non-ovariectomized (intact + sham-ovar-
iectomized) Hsd rats, an incidence comparable to the rate
(one in eight) observed in women. These results suggest that
late ovariectomy prevents development of spontaneous age-
dependent mammary tumors.

3.2. Hormone receptor expression of mammary
carcinomas

It is not known whether spontaneous mammary
carcinomas from outbred rats express ER. We investigated
whether the two spontaneous rat mammary carcinomas that
developed in Hsd rats expressed ER or other molecular
biomarkers found in breast cancer of older women. Fig. 2
shows the histological and molecular features of an invasive
cribiform adenocarcinoma that appeared at 583 days of age
(19 months) in an intact Hsd rat. This tumor expressed
nuclear ER, PR and the ER target cyclin D1. The second
carcinoma also expressed cyclin D1 but was ER+/PR–.
Thus, spontaneous carcinomas arising in aging Hsd rats have
pathological and molecular characteristics similar to those
of breast tumors found in older women. These results
suggest that the molecular mechanisms leading to age-
related mammary carcinomas in rats are similar to those
involved in the development of hormone-dependent breast
cancer in humans.

3.3. Spontaneous mammary tumor development over
life span

Although in our first experiment no spontaneous
mammary tumors were observed in the late ovariectomized
group until 110 weeks of age, it was possible that late
ovariectomy may have increased the latency of spontaneous
mammary tumor development until after 110 weeks of age.
Therefore, another experiment was conducted to measure
mammary tumor-free survival over the life span of the rats.
As shown in Fig. 3, no tumors were observed in
ovariectomized animals over their life span. The difference
in mammary tumor incidence between sham-ovariecto-
mized and ovariectomized animals was significant
(\( P = 0.0358 \)). Tumor multiplicity (0.76 tumors/rat) and
incidence of mammary carcinoma (14.28%) obtained in
this experiment were comparable to the previous 110-week
experiment. Interestingly, maximum life span of ovariecto-
mized animals in this experiment was 42 months while for
sham-ovariectomized it was less than 30 months. However,
as the endpoint of this experiment was mammary tumor
formation and rats were euthanized when a palpable tumor
reached 2 cm of diameter, overall survival curves were not
compared. A different experimental design was required to
determine the effect of late ovariectomy on overall survival.
Nevertheless, removal of ovaries before middle age onset
can successfully prevent the development of spontaneous mammary tumors over the life span of Hsd rats.

3.4. Effect of ovariectomy on overall survival

Recently, studies on the effect of oophorectomy in women had suggested that mortality was significantly higher in women who had received prophylactic bilateral oophorectomy before age 45 [23] or 65 [24]. Therefore, it was important to determine the impact of ovariectomy on survival of Hsd rats. To evaluate overall survival, instead of euthanizing rats upon tumor presentation, tumors were surgically excised when possible. To compare our survival data with a previous study on the role of ovariectomy upon longevity [25], we also utilized rats whose ovaries were removed around 1 month of age (early ovariectomy). In this way, our data could also be compared with the more standard intervention protocol used in toxicological studies that have shown the impact of hormonal therapies in development of spontaneous mammary tumors [14,26].

In this experiment, we first compared spontaneous mammary tumor development. Again, ovariectomy, whether performed early or late in life, decreased the development of spontaneous mammary tumors when compared to sham-ovariectomized or intact controls (Fig. 4). Only one benign tumor was found upon necropsy in an ovariectomized rat (one out of 12) that died at 33 months of age (142 weeks of age).

In contrast, 29 spontaneous tumors developed in 18 non-ovariectomized rats (95% inhibition). While the incidence of mammary carcinoma was still comparable to the preceding experiment (16.67%), tumor multiplicity was significantly increased ($P = 0.002$) to 1.61 tumors per rat (Table 1) due to surgical excision of tumors. From 11 successful surgeries (i.e., rats survived for at least 15 days after surgery), eight rats (73%) developed at least one additional mammary tumor. The maximum number of tumors observed per rat was four. Thus, surgical excision of palpable tumors can increase the total number of spontaneous mammary tumors per rat, and improved the ability to detect significant differences in spontaneous mammary tumor development, when testing novel preventive strategies.

To compare overall survival, groups whose surgical intervention procedure (sham-ovariectomized or ovariectomized) was done early, were combined with the late intervention groups because survival curves for the same intervention were similar. The 110-week survival rate for non-ovariectomized animals was 55.5% while for ovariectomized
animals was 91.66% \( (P = 0.0492) \). Overall survival (Fig. 5) was also significantly different \( (P = 0.0121) \). Median survival increased from 810 days for non-ovariectomized animals to 888 for ovariectomized animals. Thus, in Hsd rats, ovariectomy did not impair overall survival.

In summary, overall mammary tumor incidence was 73.81% for non-ovariectomized animals. Although most tumors were benign (Table 2), 6 out of 42 (14.29%) non-ovariectomized animals had spontaneous mammary carcinomas. Late ovariectomy decreased the incidence of spontaneous mammary tumor development to 5.26% (relative risk = 0.05, 95% CI = 0.0072–0.354, \( P < 0.0001 \)) while no mammary carcinomas were observed (Table 2). Thus, this animal model may enable the testing of novel prevention strategies that, as shown here for late ovariectomy, can efficiently prevent development of spontaneous mammary tumors.

### Table 1
Number of spontaneous mammary tumor per rat (multiplicity)

<table>
<thead>
<tr>
<th>Group ( (n) )</th>
<th>No. of MT</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Ovx w/out MT Exc (24)</td>
<td>18</td>
<td>0.75</td>
</tr>
<tr>
<td>Non-Ovx w/MT Exc (18)</td>
<td>29</td>
<td>1.61*</td>
</tr>
<tr>
<td>Early ovariectomy (6)</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Late ovariectomy (19)</td>
<td>1</td>
<td>0.05**</td>
</tr>
</tbody>
</table>

\( *P = 0.0023, **P = 0.0003, \) significantly different from non-ovariectomized (Ovx) w/out mammary tumor excision (MT Exc); No: number.

### Table 2
Histopathology of spontaneous mammary tumors

<table>
<thead>
<tr>
<th>Group ( (n) )</th>
<th>Fibroadenoma</th>
<th>Adenoma</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (18)</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sham-ovariectomized (24)</td>
<td>20</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Early ovariectomy (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Late ovariectomy (19)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4. Discussion

In this study we evaluated whether late ovariectomy (before middle age onset) was able to prevent development of spontaneous mammary tumors in Hsd rats. Our data clearly indicates that late ovariectomy can efficiently prevent development of spontaneous, benign and malignant mammary tumors in Hsd rats. This rat model mimics the human situation such that age-dependent ER-positive breast carcinomas arise spontaneously, without prior treatment with a known carcinogen, at a frequency of one in eight women.

Currently, prevention trials for hormone-dependent breast cancer, such as the National Surgical Adjuvant Breast and Bowel Project P-1 (P-1) or P-2 trial needed to enroll thousands of women for at least 5 years [2,4]. In the P-1 trial, tamoxifen also decreased the risk of benign disease and the need for biopsies [27], a considerable sociological and socioeconomical problem today. Therefore, the use of a Hsd rodent model, using spontaneous mammary tumor (benign and malignant) development as surrogate for breast cancer, could be instrumental in testing new preventive strategies for ER-positive breast cancer before they are tested in women. Although the incidence of mammary carcinoma in this outbred rat is low, it is comparable to that observed in humans. The high number of benign mammary
tumors spontaneously developing may permit efficient testing of the effects of different preventive strategies in blocking development of spontaneous mammary tumors. Nevertheless, it is possible that diet or other interventions may reproducibly increase the incidence of spontaneous age-dependent mammary carcinomas [12]. Therefore, this model could be used not only to test preventive strategies but also to determine the impact of diet, hormones and other factors in the development of spontaneous mammary tumors.

Several studies using rats have already shown that agents, such as tamoxifen or the aromatase inhibitor fadrozole, when given for 2 years or less, can drastically reduce development of spontaneous benign and malignant mammary tumors in female Sprague–Dawley rats [14,26]. In most animal studies, hormonal interventions begin when the animals are young (~1–2 months of age). To our knowledge, only one study has evaluated tamoxifen treatment for 40 weeks in aged animals (56 weeks old) [14]. Similar to our results, a significant decrease in the incidence of spontaneous benign and malignant mammary tumors was observed at the end of tamoxifen treatment and, although of less magnitude, at the end of their life span. Thus Sprague–Dawley rats could be used to test specific interventions (dosage, schedule and combination strategies) in middle-aged individuals or older that can modify the risk of spontaneous hormone-dependent mammary carcinoma.

We prevented spontaneous mammary tumor formation in female Hsd rats by removing their ovaries before 7 months of age, when age-related changes in estrous cycles are evident. Young adult female Sprague–Dawley rats have a 4-day estrous cycle [22,28]. However, when they reach middle age (7–9 months), 32% of Sprague–Dawley rats start experiencing irregular cycles (4–7 days). By 15 or 16 months of age, all female rats have irregular estrous cycles [29,30]. These age-dependent irregularities in the estrous cycle differ from humans in having increased, rather than decreased, blood estrogen levels (constant estrous or pseudopregnancy). However, in the last third of their life, when most of the mammary tumors appeared, most female rats develop atrophic ovaries and become anestrous [30,31]. Therefore, the hormonal milieu in rats developing mammary tumors may be comparable to that in postmenopausal women. In women, breast cancer risk has been associated with aberrant lobular involution [32]. Complete involution is associated with reduced breast cancer risk [32]. Thus, it is possible that removal of ovaries before middle age prevented spontaneous mammary tumor development by inducing mammary gland involution. Nevertheless, our results suggest that the presence of estrogen during middle age or later is crucial for spontaneous mammary tumor development in Hsd rats.

Interestingly, 110-week survival rates and maximum life span were increased for ovariectomized animals as observed in a previous study [18]. However, there is also evidence that ovariectomy can decrease the life span of rats [25]. The difference in outcomes may be due to the use of a different strain of rats. Nevertheless, although development of spontaneous mammary tumors was inhibited in ovariectomized animals, we noticed that several ovarietomized animals died, after 110 weeks of age, due to chronic renal failure. Chronic renal failure is a common cause of unscheduled deaths for Hsd males but not for Hsd females [19]. Moreover, estrogen-deficiency in rats has been shown to accelerate progression of renal disease [33]. Thus, although late ovariectomy can decrease spontaneous mammary tumor formation, novel preventive strategies are needed which can inhibit age-dependent increases in breast cancer risk without affecting the normal function of other organs.

In women, bilateral ovariectomy before age 45 [34] or 50 [35] can reduce breast cancer risk. However, another study, observing an increase in mortality when bilateral oophorectomy was done before age 45 but not after 45 years of age, found an increased risk of estrogen-related cancer [23]. As the authors suggested, it was possible that the population undergoing bilateral oophorectomy may have had certain features leading to their increased mortality [23]. Nevertheless, to investigate the impact of ovariectomy at different ages on risk of spontaneous mammary carcinoma development, other age-related processes, and longevity in an animal model, further studies will be needed with a larger number of rats. Nonetheless, this rat model can be used to identify novel preventive interventions that may inhibit spontaneous age-related mammary tumor development and, at the same time, may increase overall survival.

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Conflict of interest

None.

References


