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Anti-oestrogenic and Anti-tumour Properties of Prolonged Tamoxifen Therapy in C3H/OUJ Mice

V.C. Jordan, Mary K. Lababidi and Dawn M. Mirecki

The anti-oestrogenic and anti-tumour properties of a sustained-release preparation of tamoxifen were evaluated in female C3H/OUJ mice. Tamoxifen decreased uterine weight compared with controls in intact mice but caused an initial uterotrophic response for 2 months in ovariectomized mice. Prolonged tamoxifen therapy in ovariectomized mice resulted in a uterine weight no different from controls, but these uteri were eventually (at 6 months) refractory to oestradiol. Spontaneous mammary tumours were detected in female mice between 6 months and 1 year of age during continuous cycles of pregnancy and weaning. A similar tumour frequency occurred after one cycle of pregnancy and weaning initiated at 3½ months. Prolonged tamoxifen, started at 3½ or 4½ months of age (following pregnancy/weaning), reduced the appearance of tumours. Similarly ovariectomy at 3½ months prevented mammary tumorigenesis and prolonged tamoxifen could not increase tumour incidence consistently in ovariectomized mice. Although tamoxifen is oestrogenic in short-term tests the compound has the properties of an anti-oestrogen during prolonged administration.
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INTRODUCTION

SPONTANEOUS mammary tumours often occur in inbred mice. The cause was identified by Bittner [1, 2] as the mouse mammary tumour virus, which is transferred to offspring via mother's milk. The tumour frequency in female mice can be decreased by early ovariectomy [3]. Oestrogen is linked to tumorigenesis because male mice do not generally acquire mammary tumours but tumours can be induced by oestrogen injections [4]. This observation led Lacassagne [5] to predict that an agent could be developed that would antagonize the actions of oestrogen in the mammary tissue and prevent tumours. Hence, after some fifty years, tamoxifen, which is used to treat breast cancer [6], has been advocated as a prophylactic in women at high risk [7–9]. The concept has some merit.

Our goal was to study whether tamoxifen could prevent spontaneous mouse mammary tumorigenesis. However, tamoxifen is oestrogenic in short-term assays in mice [10, 11] and tumorigenesis might be increased.

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MATERIALS AND METHODS

Mice

Mature male and female C3H/OUJ mice were obtained from the Jackson Laboratories. A breeding colony was established to provide females. Animals were given food and water freely. Ovariectomy was done on large groups for some experiments after ether anaesthesia. Mice were used for uterotrophic experiments 2 weeks after operation.

Drug delivery

Oestradiol-17β pellets (1.7 mg oestradiol in a cholesterol pellet designed to release the steroid for 8 weeks) were obtained in one batch from Innovative Research of America, Toledo, Ohio. Mean serum levels of oestradiol (pg/ml, $n = 4$) were 5141 (S.E. 432) in week 1, 3130 (272) in week 2, 3113 (436) in week 4 and 1738 (137) in week 8. A 6-week period of oestradiol treatment was selected to be within the limits of pellet variation. Tamoxifen as free base was given by ICI and about 28 mg was administered in 2 cm silastic capsules (1.99 mm internal and 3.13 mm external diameter; Dow Corning, Midland, MI), which released drug at approximately 125 μg per day for at least 6 months.

Effect of tamoxifen on the uterus

These experiments used 2-month-old mice. In the first experiment mice were divided into groups of 7. Animals were anaesthetized with ether and a silastic implant of tamoxifen or control was inserted subcutaneously on the back. One group was killed at the start of the experiment and groups of test or control animals were killed 1, 2, 3, 4, 8 and 16 weeks later. Uteri were removed, blotted and weighed wet. In the second experiment, ovariectomized mice were divided into groups of 4 and implanted with either sustained-release tamoxifen or a control silastic capsule. Groups of tamoxifen-treated or control animals were killed at 1, 2, 3, 4, 8, 16 and 24 weeks after the start of the experiment. In the third experiment, ovariectomized mice were divided into groups of 6 and implanted with either sustained-release tamoxifen or a control capsule. A group of controls was killed at 2 months and a group of test or control animals was implanted with a cholesterol pellet containing oestradiol. These groups and a group of animals treated with tamoxifen or control, but not with oestradiol, were killed 6 weeks later. This procedure was repeated at 16 and 24 weeks.

Tumorigenesis

Groups of female mice were bred continuously from 2 months of age throughout the first year of life. Mice went through pregnancy/weaning cycles of 6 weeks between being presented with the males. The tumour frequency in the groups (A, $n = 60$; B, $n = 35$; C, $n = 35$) was recorded. In a separate experiment the effect of a single pregnancy/weaning cycle at $3\frac{1}{2}$ months of age ($n = 26$) on tumour occurence during the first year was compared with that in virgin mice ($n = 39$).

Antitumour effect of tamoxifen

Mice went through a single pregnancy/weaning cycle at 2 months of age. In the first experiment one group of animals was implanted with silastic capsules of tamoxifen 2 weeks after weaning and, in another, tamoxifen was implanted 5 weeks later. Tumour incidence was compared with that in animals implanted with silastic capsules alone. In the second experiment mice were ovariectomized 1 week after weaning and tamoxifen was implanted 1 and 4 weeks later. Tumour occurrence was compared with that in intact animals implanted with silastic capsules alone. The growth rate of each tumour was monitored and a piece was histologically examined at necropsy.

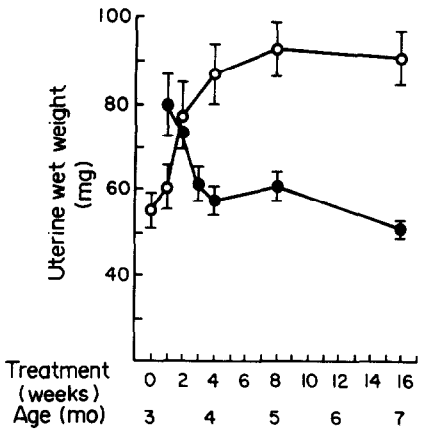

Fig. 1. Effect of sustained-release tamoxifen on uterine wet weight (mean, S.E.) of intact C3H/OUJ mice. ○ = control and ● = tamoxifen; $n = 7$ per group. Tamoxifen-treated groups were significantly different from controls ($P < 0.03$) except at 2 weeks.

Statistics

The differences in uterine weights were analysed by t test and the significance of the treatments in the tumour studies was established by Z tests.

RESULTS

Oestrogenic/anti-oestrogenic effects of tamoxifen

In the first experiment in intact mice, uterine weights rose as the control animals reached maturity (Fig. 1). The tamoxifen-treated animals had a small early rise in uterine weight but after several weeks the uterine weights fell, indicating an anti-uterotrophic effect against endogenous oestrogen. Tamoxifen had an initial uterotrophic action in ovariectomized mice (Fig. 2). However, as therapy continued, uterine weight declined so that after 4 months the control and tamoxifen-treated uterine weights were similar.

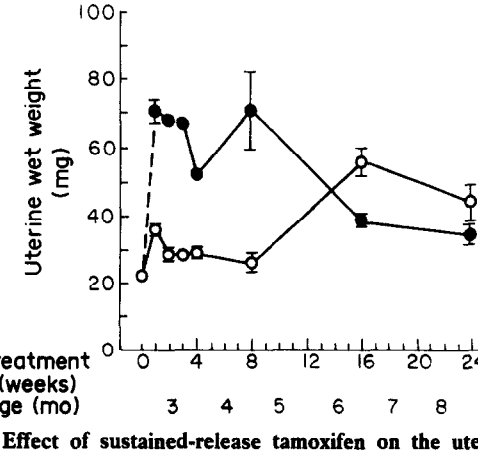

Fig. 2. Effect of sustained-release tamoxifen on the uterine wet weight (mean, S.E.) of ovariectomized mice. ○ = control and ● = tamoxifen-treated; $n = 4$ per group. Tamoxifen was significantly uterotrophic by 1 week ($P = 0.0003$) but was not significantly different from control at 24 weeks.

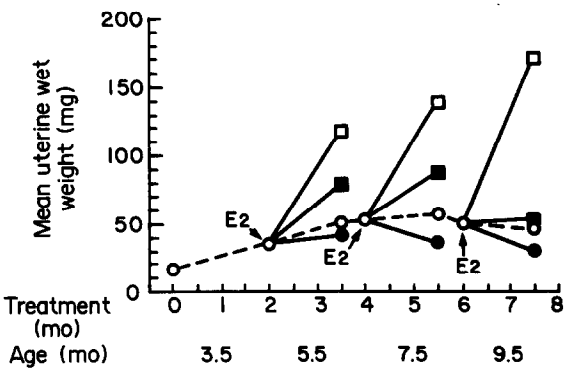

Fig. 3. Anti-oestrogenic effect of sustained-release tamoxifen implanted into ovariectomized mice (details in Materials and Methods). Each group = 4–6 mice. Significant differences (at least $P < 0.02$) at 3½, 5½ and 7½ months of treatment existed, although the group treated with tamoxifen and challenged with oestradiol was no different from control at 7½ months. ○ = controls killed at the indicated times; ● = mice treated with tamoxifen alone; □ = implanted with oestradiol pellets and killed 6 weeks later; and ■ = treated with tamoxifen and implanted with oestradiol pellets and killed 6 weeks later.

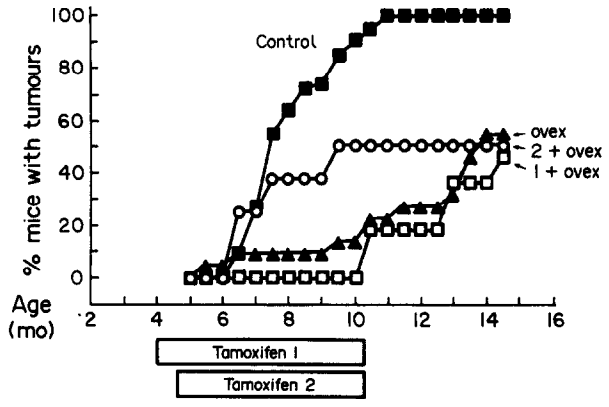

Fig. 5. Effect of sustained-release tamoxifen on appearance of mammary tumours in ovariectomized mice (▲, $n = 22$). ■ = Animals were implanted with tamoxifen at week 1 (tamoxifen 1, $n = 11$) and ○ = at 4 (tamoxifen 2, $n = 7$) weeks after ovariectomy. Treatments were significantly different from controls (at least $P < 0.001$) at 14½ months but individual treatments were not significantly different from each other.

These data, however, do not indicate whether the drug is affecting the uterus. The experiment was repeated, but control and tamoxifen-treated mice were subjected to oestradiol challenge (implanted oestradiol pellets) at 2, 4 and 6 months. Tamoxifen inhibited oestradiol-stimulated increases in uterine wet weight (Fig. 3). However, the longer the therapy with tamoxifen was continued, the greater was the refractoriness of the uterus to oestradiol. Untreated (control) uteri were extremely sensitive to stimulation with oestradiol.

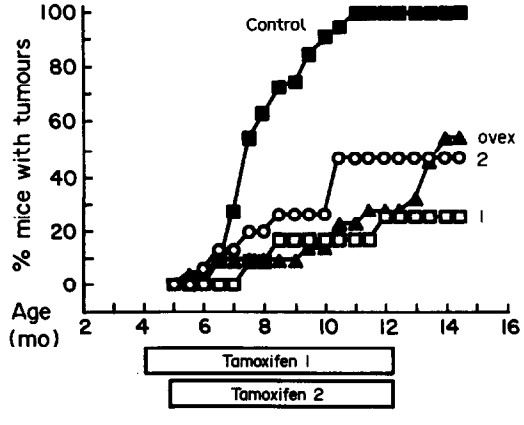

Fig. 4. Effect of sustained-release tamoxifen on appearance of mammary tumours. ■ = control, $n = 11$; □ = intact animals were implanted with tamoxifen at 2 weeks (tamoxifen 1 $n = 11$) or ○ = at 5 weeks (tamoxifen 2, $n = 15$) after weaning (at approximately 3½ months of age). ▲ = ovariectomy (ovex) 1 week after weaning ($n = 22$). Treatments were significantly different from controls (at least $P < 0.001$) at 14½ months but individual treatments were not significantly different from each other.

Frequency of mammary tumours

The first appearance of mammary tumours in the groups bred continuously was similar (data not shown). Mammary tumours started to appear in all groups between 6 and 7 months of age and nearly all the animals had tumours by 12 months. In contrast, tumours appeared more slowly in virgin animals (data not shown), but this process was accelerated by one pregnancy/weaning cycle at 3½ months of age.

Obviously, since tamoxifen is an anti-fertility agent in the mouse [12] a model that required continuous pregnancy would be impractical to study anti-tumour properties. However, because a single pregnancy produced an optimal tumour frequency, we considered this would be a suitable model to test the effect of subsequent tamoxifen therapy upon the appearance of mammary tumours.

Anti-tumour effects of tamoxifen

Prolonged release preparations of tamoxifen, implanted at 2 and 5 weeks after weaning, inhibited mammary tumorigenesis (Fig. 4). Later implantation of tamoxifen was less effective. After administration to ovariectomized mice to determine whether the oestrogenicity of the drug could facilitate tumorigenesis, ovariectomy decreased the appearance of tumours but the addition of tamoxifen produced a slight increase in tumour occurrence. Nevertheless, the drug did not increase the frequency to that observed in intact animals (Fig. 5).

DISCUSSION

Tamoxifen can produce complete anti-oestrogenic effects in the mouse uterus and this action may be responsible for the inhibitory effect upon mammary tumorigenesis. Paradoxically, tamoxifen is classified as an oestrogen in short-term vaginal and uterine assays in the mouse [10–12] although Pavlik *et al.* [13] demonstrated some anti-uterotrophic activity for tamoxifen and its potent metabolite monohydroxytamoxifen in uterine wet weight tests in immature female mouse. However, the effect was noted only at low doses. In contrast, a large depot injection of tamoxifen (3 mg) causes an initial oestrogenic response with vaginal cornification in the ovariectomized mouse which is then followed by several weeks of leucocytic smears and the vagina is

refractory to the effects of oestrogen [14, 15]. We also found that an increase in uterine weight that declined over the next 10 weeks [15]. The present study confirmed this observation, but the finding that tamoxifen made the uterus refractory to oestrogen stimulation the longer therapy is continued is interesting. A similar effect was seen in the athymic mouse after 6 months of tamoxifen [16]. However, a differential anti-oestrogenic effect occurred in the xenograft model. Long-term tamoxifen therapy caused the uterus to be refractory to subsequent oestrogen stimulation; however, the growth of transplanted human breast tumours derived from MCF-7 cells is reactivated by oestrogen [16].

These results in the mouse may have clinical implications. Tamoxifen therapy for breast cancer produces early oestrogen-induced changes in vaginal cytology [17]. There is, however, no information about the effects of long-term adjuvant tamoxifen therapy on vaginal cytology. Similarly, there is no information about the action of such therapy on uterine tissue. The current fashion of an extended therapeutic regimen [18–20] necessitates investigation of uterine histology to determine whether prolonged stimulation of endometrium occurs. It would seem to be an advantage to have a quiescent, rather than a proliferating, endometrium to avoid the potential endometrial problems that were associated with postmenopausal oestrogen replacement therapy [21]. Fornander *et al.* [22] showed that long-term adjuvant tamoxifen therapy controlled the appearance of second primary breast cancers but increased the frequency of endometrial carcinomas, a result consistent with our finding [23] that tamoxifen inhibited the growth of an implanted MCF-7 breast tumour whilst stimulating the growth of a human endometrial tumour implanted in athymic mice. In the present study tamoxifen inhibited mammary tumorigenesis and the effects of oestrogen in the uterus.

Increased tumorigenesis in mice was observed with diethylstilbestrol [4] but we have now demonstrated that tamoxifen is not sufficiently oestrogenic to promote tumorigenesis. Tamoxifen and monohydroxytamoxifen retard the growth of pregnancy-dependent tumours transplanted into mice [24, 25]. Our present study supports Lacassagne's prediction [5] that an oestrogen antagonist would be found which decreased the appearance of spontaneous mouse mammary tumours.

Overall, these studies in the C3H/OUJ mouse are consistent with the anti-oestrogen inhibiting the early promotional stages of carcinogenesis that is facilitated by ovarian steroids. This result is similar to data from carcinogen-induced rat mammary tumour models that demonstrate the effectiveness of long-term tamoxifen therapy in inhibiting the appearance of tumours after the carcinogenic insult [26].

In breast cancer we do not know the cause or timing of the carcinogenic insult. It is therefore unlikely that an anti-oestrogen could be used prophylactically. It is possible, however, to foresee the use of an anti-oestrogen as chemosuppressive [27], preventing oestrogen-driven tumour cell replication after malignant transformation. Implementation of such a strategy would be unwise until rigorous clinical and laboratory evaluation was complete.

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