Reversal of the Antitumor Effects of Tamoxifen by Progesterone in the 7,12-Dimethylbenzanthracene-induced Rat Mammary Carcinoma Model

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ABSTRACT

Coadministration of progesterone (4 mg/day) opposed the antitumor activity of tamoxifen (100 μg/day) in rats bearing 7,12-dimethylbenzanthracene-induced tumors and also partially prevented the inhibition by tamoxifen (50 μg/day started 30 days after 7,12-dimethylbenzanthracene administration) of tumor occurrence even after tamoxifen therapy had been established for 1 or 2 mo. Although prolonged progesterone treatment raised progesterone levels, serum total estrogen levels were not raised above control. The reversal by progesterone of the inhibition of tumor occurrence produced by tamoxifen was blocked by the antiprogestin RU 486. These results demonstrate that progesterone can reverse the tumorstatic action of tamoxifen in the 7,12-dimethylbenzanthracene-induced tumor model and that this may be via a progesterone receptor-mediated mechanism.

INTRODUCTION

The antiestrogen tamoxifen has become the antisteroidal therapy of choice for the treatment of hormone-dependent breast cancer (1). However, although tamoxifen is as effective as the previously used ablative and additive therapies (1), failure ultimately occurs, even in cases where tamoxifen has produced a long initial period of tumor remission (1). A number of possible mechanisms resulting in tumor relapse have been proposed (2), but at present the main cause remains unclear. Combinations of tamoxifen with other forms of antihormone therapy have not been shown to have any clinical advantage over tamoxifen alone (1). This suggests that, during tamoxifen therapy, tumors lose the requirement of hormone stimulation to maintain tumor growth. However, contrary to this are the reports that more than half the patients who initially respond to tamoxifen alone and subsequently relapse have objective remission following hypophysectomy, androgen therapy (3), or aminoglutethimide treatment (4, 5).

Recently the sequential use of tamoxifen and progestin has been investigated (6, 7). The rationale for this is that tamoxifen has been observed to increase the concentration of progesterone receptors in breast tumors (8, 9) and thus may prime or sensitize the tumor to high-dose progestin therapy.

However, the increase in progesterone receptor produced by tamoxifen could also be involved in the therapeutic failure of this antiestrogen. Low doses of progesterone have been demonstrated to produce a positive stimulus in rat mammary tumors induced by carcinogens (10, 11) or in the transplanted MTW9 carcinoma (12). Furthermore, in the mouse, progesterone has been implicated as a factor in the development of spontaneous mammary tumors during pregnancy (13, 14) and mammary tumors induced in inbred strains by hypophyseal isografts (15). The combination of estrone and progesterone has been demonstrated to induce tumors in ovariectomized GF mice, whereas neither is effective alone (16).

The progestational content of the contraceptive pill has been related to the risk of breast cancer in humans (17, 18). Although this finding is controversial (19), the combination with animal data is suggestive that progesterone may play a part in breast cancer growth.

The increase in progesterone receptor number produced by tamoxifen (6, 7) could therefore prime for endogenous progestins and result in tumor stimulation. In support of this hypothesis is the report that low doses of progestin therapy in combination with tamoxifen are less effective for breast cancer therapy than tamoxifen alone (20). Furthermore, consistent with this hypothesis, tumors failing tamoxifen therapy would still be hormone dependent, and thus the reports of objective responses produced by other endocrine therapies (3–5) after cessation of tamoxifen treatment would be explained.

In order to investigate these observations we examined the influence of progesterone on tamoxifen therapy of mammary tumors in rats exposed to DMBA.

MATERIALS AND METHODS

DMBA and progesterone were obtained from the Sigma Chemical Company (St. Louis, MO). Tamoxifen was a gift from Stuart Pharmaceuticals (Wilmington, DE), and RU 486 was a gift from Roussel UCLAF (Romainville, France).

Drug administration was by s.c. injection into the loose fold of skin on the back of the neck. Progesterone and tamoxifen solutions were prepared by adding a small volume of ethanol to crystalline material and then stirring with peanut oil. The progesterone solution was warmed to aid solubility. The ethanol in the tamoxifen solution was evaporated under N2 once in solution; however, the ethanol (3% v/v) was required in the progesterone solution to prevent crystallization. RU 486 was prepared as a microcrystalline suspension in peanut oil which was vigorously mixed before injection. DMBA, dissolved in peanut oil by stirring overnight, was administered (20 mg in 2 ml) by gavage to 50-day-old female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) as previously described (21).

Progesterone Interaction with the Inhibition of Tumor Growth by Tamoxifen. Animals from a pool of rats treated with DMBA were randomly entered into 4 groups when tumors reached measurable size (diameter, >1 cm). Rats were entered into the groups between 80 and 150 days after DMBA. The 4 groups were treated daily for the duration of the experiment with (a) peanut oil, (b) 4 mg progesterone, (c) 100 μg tamoxifen, and (d) 4 mg progesterone and 100 μg tamoxifen. Tumor sizes were determined for 11 wk by caliper measurements and calculated using the formula, length/2 × width2/2 × π.

Progesterone Interaction with the Inhibition of Tumor Development by Tamoxifen. Thirty days after DMBA administration, rats were randomized into groups of 25 animals. In an initial experiment 4 groups were treated daily with (e) peanut oil alone, (f) 4 mg progesterone, (g) 50 μg tamoxifen, and (h) 4 mg progesterone and 50 μg tamoxifen. Treatment was given for about 12 wk. Animals were palpated at weekly intervals. Groups g and h were followed for about 16 wk after treatment stopped.

In a further study, 4 groups were treated daily with peanut oil alone (i) or containing 50 μg tamoxifen (j, k, and l). After 1 mo, Group j

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3 The abbreviation used is: DMBA, 7,12-dimethylbenzanthracene.
received additional treatment with 4 mg progesterone. After 2 mo, Group A received additional treatment with 4 mg progesterone. Animals were palpated at weekly intervals.

The Influence of an Antiprogestin on Progesterone Interaction with the Inhibition of Tumor Development by Tamoxifen. Thirty days after DMBA administration, 100 rats were randomized into 4 groups of equal size and treated with peanut oil alone (m) or containing 50 µg tamoxifen (n, o, and p). After 1 mo, Group o received additional treatment with 4 mg progesterone, and Group p received additional treatment with 4 mg progesterone and 5 mg RU 486. Animals were palpated at weekly intervals to detect tumors.

Hormone Levels and Uterine Weights. Circulating hormone levels and uterine weights were measured in rats chronically treated (approximately 12 wk) with peanut oil, tamoxifen (50 µg/day), or tamoxifen (50 µg/day) and progesterone (4 mg/day). Blood samples were taken approximately 4 h after the final injection by ether anesthesia and bleeding from the abdominal aorta regardless of the stage in the estrus cycle. Blood was allowed to clot overnight at 4°C and then centrifuged at 2000 × g, and the serum was removed and stored at −20°C until analyzed. Progesterone and total estrogen measurements were made using commercially available radioimmunoassays designed for human samples. Progesterone was measured on 50-µl serum samples by the progesterone coat-a-count (Diagnostic Products, Los Angeles, CA), ovariectomized rat serum was used as a test control, and spiked samples were used to validate the assay (data not shown). Total estrogen measurements were made on 300-µl serum samples using RSL 125I-labeled total estrogen radioimmunoassay (Radioassay Systems Laboratories, Inc., Carson, CA). This assay had a low standard of 5 pg/ml, and the intraassay variation at 53 pg/ml was given as a coefficient of variation of 5.5%.

Statistical Analysis. The rate of change of tumor size between groups was analyzed using analysis of variance decomposition (22). The Student t test was used for other comparisons. Significance was recognized if P < 0.05.

RESULTS

Progesterone Interaction with the Inhibition of Tumor Growth by Tamoxifen. The administration of tamoxifen (100 µg/day) to tumor-bearing rats produced a reduction in tumors to <20% of their initial size in 10 of 15 tumors and stasis of growth in a further 3 tumors (see Fig. 1C). This contrasts to the peanut oil-treated control animals in which only 5 of 16 tumors noticeably decreased in size, and the majority of tumors grew (see Fig. 1A). In the progesterone-treated group a similar pattern of tumor progression to that of the control group was observed but with fewer regressing tumors (see Fig. 1B). The combinations of tamoxifen and progesterone produced a tumor response midway between the tamoxifen alone- and progesterone alone-treated groups. Tumor regression in the combined treatment group not only occurred in fewer tumors than that of the tamoxifen alone group (8 of 18), but also their regression was less rapid. Furthermore, 7 of the 18 tumors grew very rapidly (see Fig. 1D). Analysis of the rate of change of tumor size (change in size divided by time span from assignment to necropsy) between treatment groups using analysis of variance decomposition shows a significant interaction between tamoxifen and progesterone (P < 0.05) with progesterone partially reversing the tumor regression produced by tamoxifen.

Progesterone Interaction with the Inhibition of Tum oral Development by Tamoxifen. The treatment with progesterone (4 mg/day) 30 days after DMBA administration resulted in more (see Fig. 2) and larger tumors (see Fig. 3) developing in the following 8 wk than in the control group. In contrast, tamoxifen (50 µg/day) treatment over this same period almost totally prevented tumor occurrence and development (see Figs. 2 and 3). The tumor burden in the tamoxifen-treated group was significantly different after 8 wk of treatment when compared to control (P < 0.0001). This antitumor action of tamoxifen was reversed by the simultaneous administration of progesterone which resulted in significantly (P < 0.01) more and larger tumors per rat (see Figs. 2 and 3) and more rats developing tumors than the tamoxifen alone group.

Cessation of tamoxifen treatment alone after 13 wk resulted in a loss of the protective antitumor action and the development of tumors over the next 16 wk (see Fig. 2). Similarly, stopping progesterone combined with tamoxifen resulted in tumor de-
weeks from start of treatment

Fig. 2. Effect of tamoxifen and progestosterone treatment alone or in combination on the occurrence of rat mammary tumor induced by DMBA. Rats were administered DMBA 30 days prior to drug treatment. Groups of 25 animals were injected (s.c.) daily with tamoxifen (50 μg), progestosterone (4 mg), or tamoxifen (50 μg) and progestosterone (4 mg). All drugs were given separately in peanut oil (0.1 ml). Control (O) animals received peanut oil alone. Tumor burden was calculated as the number of tumors per group divided by the number of rats. Treatment period is indicated by the hatched box. Treatment was stopped in the tamoxifen and tamoxifen plus progestosterone groups at the time indicated by the arrow.

Fig. 3. Tumor sizes 8 wk after the start of treatment on animals described in Fig. 2. Tumors that were too small to measure (<1 cm²) were classified descriptively. Tumor numbers in each group are shown in brackets. CONT., control; PROG., progestosterone; TAM., tamoxifen.

Influence of an Antiprogestin on the Interaction of Progestosterone on the Inhibition of Tumor Development by Tamoxifen. The administration of tamoxifen for 1 mo established a therapy that prevented tumor formation. As previously shown the coadministration of progestosterone reversed the inhibition. The further addition of the antiprogestin, RU 486 (5 mg/day), was significant (P < 0.05) in preventing the reversal by progestosterone (4 mg/day) of the antitumor action of tamoxifen (50 μg/day) after 6 wk of treatment (see Fig. 5). This prevention of tumor formation by RU 486 was to a level slightly but not significantly below that of tamoxifen alone.

DISCUSSION

The inhibition of DMBA tumor induction and growth by tamoxifen is well documented (23). Similarly, this model has been used extensively in studies of progestosterone action on mammary tumors (24, 25). This was, therefore, a useful model to investigate the hypothesis that progestosterone may reverse the antitumor action of tamoxifen.

The findings, that tumor regression and the protection against tumor development by tamoxifen treatment were partially reversed by the coadministration of progestosterone, support this hypothesis. However, the mechanism behind the reversal of the antitumor activity of tamoxifen by progestosterone is unclear.

Previously, Jabara (24) demonstrated progestosterone treatment caused the development of DMBA tumors in rats earlier than...
Fig. 4. Effect of progesterone treatment on the occurrence of DMBA-induced mammary tumors in rats treated with tamoxifen. Rats were administered DMBA 30 days prior to drug treatment. Groups of 25 animals were given injections (s.c.) daily with tamoxifen (50 mg) alone for 1 mo. Rats were then continued on either tamoxifen alone (50 mg) or tamoxifen (50 mg) and progesterone (4 mg) alone for 2 mo. All drugs were given separately in peanut oil (0.1 ml). Control (O) animals received peanut oil alone. Tumor burden was calculated as the number of tumors per group divided by the number of rats. Arrows indicate the time at which progesterone treatment was commenced in the combined treatment groups.

Fig. 5. Effect of RU 486 on the reversal by progesterone of the antitumor action of tamoxifen. Rats were administered DMBA 30 days prior to drug treatment. Groups of 25 animals were given injections (s.c.) daily with tamoxifen (50 mg) alone for 1 mo. Rats were then continued on either tamoxifen alone (50 mg) or tamoxifen (50 mg) and progesterone (4 mg). All drugs were given separately in peanut oil. Control (C) animals were administered only peanut oil. Tumor appearance was calculated as the number of tumors occurring during the treatment period divided by the number of rats.

The original hypothesis of progesterone reversal of the antitumor action of tamoxifen required progesterone to influence tumor growth by cellular activation via the progesterone receptor. Evidence supporting the reversal of the antitumor action of tamoxifen by progesterone being via progesterone receptor activation was obtained using the antiprogestin RU 486 to prevent this effect. However, although this argues in favor of a progesterone receptor-mediated reversal, RU 486 is not selective and has considerable antiglucocorticoid activity and moderate antiandrogen activity, and it produces a variety of endocrine-related responses (28) which may also play a role. Furthermore, this does not exclude the possibility that progesterone is influencing tumor growth by receptor binding at sites distant to the tumor. A single injection of progesterone (10 mg) has been demonstrated to induce pseudopregnancy in female rats.

in control animals and produced a faster rate of tumor growth. Similar findings were observed in this study (Fig. 2). We reasoned that the promotion of tumor growth by progesterone may result in tumors undergoing rapid development before tamoxifen treatment has attained sufficient control of tumor growth. This possibility was addressed and dismissed by the study of the influence of progesterone on tumor initiation following established tamoxifen therapy (1 and 2 mo). These findings clearly demonstrate that progesterone was acting in the presence of sufficient tamoxifen to prevent tumor development.

While reversing the tumoristatic action of tamoxifen in the mammary gland progesterone acted as an estrogen antagonist in its reduction of uterine wet weight. This type of effect on

**Table 1: Serum hormone levels and uterine weights taken from animals following daily treatment with agents for 12 wk**

<table>
<thead>
<tr>
<th></th>
<th>Total estrogen (estrone + estradiol) (pg/ml)</th>
<th>Progesterone (mg/ml)</th>
<th>Uterine wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>44.4 ± 4.4*</td>
<td>27.4 ± 7.0</td>
<td>411.4 ± 39.8</td>
</tr>
<tr>
<td>Tamoxifen (n = 7)</td>
<td>44.6 ± 2.8</td>
<td>80.6 ± 6.6</td>
<td>167.0 ± 10.8</td>
</tr>
<tr>
<td>Tamoxifen + progesterone (n = 5)</td>
<td>33.6 ± 6.7</td>
<td>79.2 ± 5.8</td>
<td>160.0 ± 11.0</td>
</tr>
</tbody>
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* Mean ± SE.
* Numbers in parentheses, number of estimates for each measurement.
and results in a period of increased prolactin production (29). Since DMBA-induced tumors are prolactin dependent (30), the raised levels of prolactin may result in increased tumor growth.

However, Kelly et al. (31) reported that chronic progestosterone (4 mg/day) treatment of rats did not significantly change circulating prolactin levels or the number of prolactin receptors on DMBA-induced tumors. The treatment did, however, cause an increase in the early incidence of these tumors.

Overall, the findings demonstrate that progestosterone can reverse the tumorigenic actions of tamoxifen in this model and that this is possibly through progestosterone receptor activation. How this may relate to the failure of tamoxifen in the human disease is uncertain; however, these findings suggest that the use of low doses of progesterational agents in patients receiving tamoxifen therapy may be inappropriate.

Indeed, following the submission of this paper for publication Hissom and Moore (32) demonstrated the activity of the progestin, R5020, to stimulate the growth of T47D human breast cancer cells in culture. These cells are known to contain a high level of progesterone receptor (33).

REFERENCES

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