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Estrogen Receptors in Androgen-induced Breast Tumor Regression[1]

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SUMMARY

The hormone-dependent 7,12-dimethylbenz(a)anthracene rat mammary tumor has been shown to regress when administered pharmacological doses of testosterone propionate. Tumor regression was correlated with estrogen receptor before and 15 to 20 days following testosterone therapy. A dramatic decline of receptor occurred in all regressing tumors, whereas those administered sesame oil alone maintained both growth and receptor content. Although receptor in regressing tumors was significantly less than in the untreated biopsies, the small amount of remaining receptor maintained the same binding affinity to estradiol, showing that testosterone affects the number and not estrogen affinity of the estrogen receptor. These studies suggest that testosterone depletion of estrogen receptor may be causally related to tumor regression.

INTRODUCTION

Pharmacological doses of androgens induce regression of 50 to 80% of DMBA[4]-induced rat mammary carcinomas (10, 22), as well as 15 to 30% of mammary carcinomas in human subjects (8, 9, 13). In the latter case, the tumor regression is reasonably well correlated with tumor ER content (13). Deshpande *et al.* (7) found that pretreatment of patients with dromostanolone propionate decreased the amount of injected [^{3}H]estradiol present in human breast tumors, compared with controls, suggesting a reduction in tumor ER. These observations were not confirmed by Braunsberg *et al.* (1), but Mobbs (14) reexamined the question in DMBA tumors and concluded that prolonged testosterone treatment might decrease the uptake of estradiol in breast tumors by altering the metabolism of the steroid. Since ER was not measured in these latter studies, we examined tumor ER levels in DMBA rat mammary tumors before and after pharmacological androgen therapy. We find that ER is markedly reduced in tumors regressing during androgen therapy.

MATERIALS AND METHODS

Mammary Tumor Induction. Mammary tumors were induced in 50-day-old virgin female Sprague-Dawley rats (Holtzman Co., Madison, Wis.) by a single dose of DMBA (Sigma Chemical Co., St. Louis, Mo.) by gastric intubation. In 2 to 4 months, mammary tumors appeared and were measured with calipers in 2 diameters, 3 times weekly. Many rats developed multiple tumors. In such cases, only one tumor was chosen to study, the remainder being excised prior to TP treatment. When a tumor reached a mean diameter of 3 to 4 sq cm, approximately one-half of the tumor was removed at diestrus. The biopsy tissue was trimmed of fat and normal tissue, frozen in liquid nitrogen, and stored at −70° until assayed. A representative section of the biopsy was removed for histological examination.

Testosterone-Induced Regression. Following biopsy, the remaining intact tumor was allowed to regrow until it resumed its original size. Rats were then given twice weekly s.c. injections of 1.2 mg TP in 0.2 ml sesame oil; control animals received sesame oil alone. Tumors were classified as regressors if they regressed to 50% of their pretreatment size. At this point, they were removed and frozen (−70°). Control tumors from sesame oil-injected animals were removed and frozen after approximately 15 days.

ER Assays. Frozen tissue was pulverized with a Thermovac tissue pulverizer (Thermovac Industries Corp., Copiague, N. Y.). Approximately 200 mg of tissue were homogenized in 1 ml of 10 mM Tris-HCl-1.5 mM EDTA-0.5 mM dithiothreitol buffer, pH 7.4 at 4°, with a Polytron PT-10-ST homogenizer (Brinkman Instruments, Inc., Westburg, N. Y.) at a speed setting of 3.5 for 3 10-sec intervals. All procedures were performed at 0-4° unless otherwise indicated. The homogenate was centrifuged 800 × *g* for 10 min, and the supernatant was saved. The pellet was twice washed by suspension in 1 ml of 10 mM Tris-HCl-1.5 mM EDTA-0.5 mM dithiothreitol and was centrifuged at 800 × *g* for 10 min. The 3 supernatants were combined and centrifuged at 105,000 × *g* for 30 min. The resultant supernatant cytosol was adjusted to 1.5 mg protein/ml of 10 mM Tris-HCl-1.5 mM EDTA-0.5 mM dithiothreitol as determined by absorption at 260 and 280 nm (11). Protein values were later assayed more accurately by the method of Lowry et al. (12).

The washed nuclear pellet was extracted once with 2 ml of 10 mM Tris-0.6 mM KCl-1.5 mM EDTA-0.5 mM dithiothreitol buffer, pH 8.5 at 4°, for 1 hr and then centrifuged at 105,000 × *g* for 30 min. The supernatant nuclear extract was adjusted to 0.25 mg protein per ml 10 mM Tris-HCl-1.5 mM EDTA-0.5 mM dithiothreitol, pH 7.4. The pellet was saved for DNA analysis (2).

Cytoplasmic and nuclear extracts were assayed with

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[4] The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; ER, estrogen receptor; TP, testosterone propionate.

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minor modifications of protamine assays previously described (4, 23). Fractions of diluted cytosol (200 μl of 1.5 mg/ml) or nuclear extract (500 μl of 0.25 mg/ml) were each incubated for 3 to 5 min with 250 μl of protamine sulfate, 1 mg/ml (Eli Lilly and Co., Indianapolis, Ind.) and the precipitate was sedimented by centrifugation at 800 × *g* for 10 min. The supernatant was decanted and the presence of estrogen receptor in the precipitate was determined either by a single dose or saturation binding assay.

For the single-dose assay, cytosol or nuclear protamine pellets were incubated for 18 hr at 0–2° with 2 to 5 nM radiolabeled estradiol [17β-[^{3}H]estradiol (Amersham Searle, Arlington Heights, Ill.)], 100 Ci/mmole, in a volume of 500 μl. Nonspecific estrogen binding was determined by a parallel incubation with a 100-fold excess of diethylstilbestrol (DES). Following incubation the supernatant was decanted and the pellets washed three times with 10 mM Tris-HCl-1.5 mM EDTA-0.5 mM dithiothreitol buffer, pH 7.4. 17 β-[^{3}H]Estradiol in the protamine pellets was extracted and counted in 5 ml of toluene scintillation fluor (4.0 g PPO, 0.05 g POPOP, 1 liter toluene) in a Beckman LS233 counter with a counting efficiency of 45%.

For saturation analysis, cytosol or nuclear protamine pellets were incubated 18 hr at 0–2° with increasing quantities of 17β-[^{3}H]estradiol (0.05 to 10 nM in a final volume of 500 μl) with or without 1 μM diethylstilbestrol to determine nonspecific estrogen binding. All other procedures were as described above. Data were analyzed by the method of Scatchard (19).

RESULTS

Tumor Growth. Chart 1, *A* and *B*, represents typical growth patterns of TP and sesame oil-injected DMBA rat mammary tumors. Approximately one-half of each growing tumor was removed (at biopsy) when the mean diameter reached 3 sq cm. After the tumor had reestablished its original size, TP therapy was initiated. All tumors in these studies that were injected with TP repressed within about 20 days, whereas those that received vehicle alone continued to grow (Chart 1*B*).

ER. Tumors were assayed for ER by Scatchard analysis if sufficient tissue was available (80%). In a small number (20%), insufficient tissue was present so the single-dose assay was used. Chart 2 compares ER values before and after TP and vehicle treatment. TP markedly reduced ER levels in regressing tumors to an average of 30% of their pretreatment value, whereas vehicle treatment did not produce any consistent effect. Also the biopsy samples showed a wide range of ER values (58 ± 9 fmoles/mg protein), which fell to the same general low level (17 ± 2 fmoles/mg protein) after TP treatment. When the data were recalculated as fmoles/mg DNA, the same reduction in TP-treated tumors was seen (data not shown).

Chart 3 shows that TP reduced the number of ER-binding sites without altering the affinity of the receptor for estrogen.

Although the tumors were removed 24 hr after the last injection of TP, we considered the possibility that ER might still be residing in the nuclei. Nuclear ER was determined by an exchange assay (23) and found to be no more than 10% of the value found in the cytoplasm (data not shown).

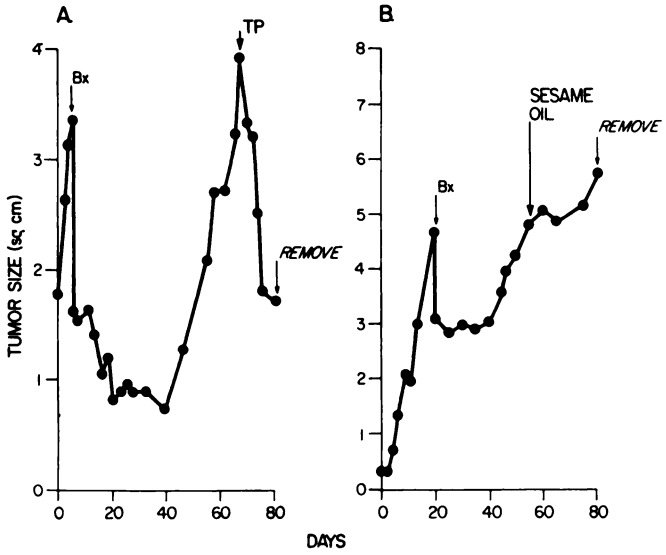

Chart 1. Growth patterns of representative DMBA-induced rat mammary tumors. When tumors reached a mean diameter of 3 to 4 sq cm, approximately one-half of each tumor was removed. When tumors resumed their original size, the rats began a course of twice-weekly injections of 1.2 mg TP in sesame oil (*A*) or sesame oil alone (*B*). Tumors were removed after 15 to 20 days. *Bx*, biopsy.

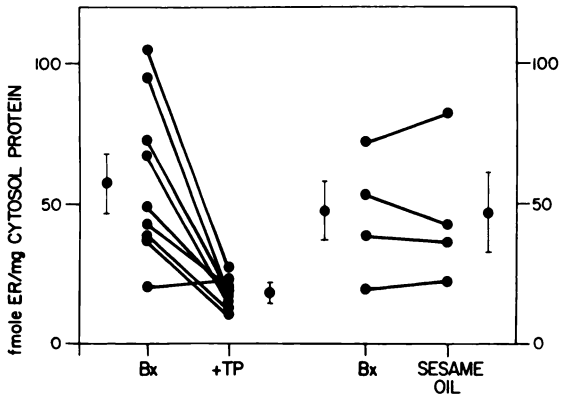

Chart 2. Estrogen receptor in DMBA rat mammary tumors before and after TP or sesame oil therapy. *Bx*, biopsy; *side points*, mean ± S.E.

DISCUSSION

We have shown that in rat mammary tumors regressing after androgen therapy, ER values are greatly reduced. An immediate question is whether the fall in ER sites is due to a specific intracellular effect of androgen or whether the general catabolic process of tumor regression decreased ER values in some nonspecific fashion. The former possibility derives some support from studies in rat uterus in which pharmacological doses of androgen translocated cytoplasmic ER to nuclear sites both *in vivo* and *vitro* (16–18, 20). Although we could not detect nuclear ER sites 24 hr after the last injection of TP, it is possible that TP did translocate cytoplasmic ER to nuclei but that nuclear processing of ER

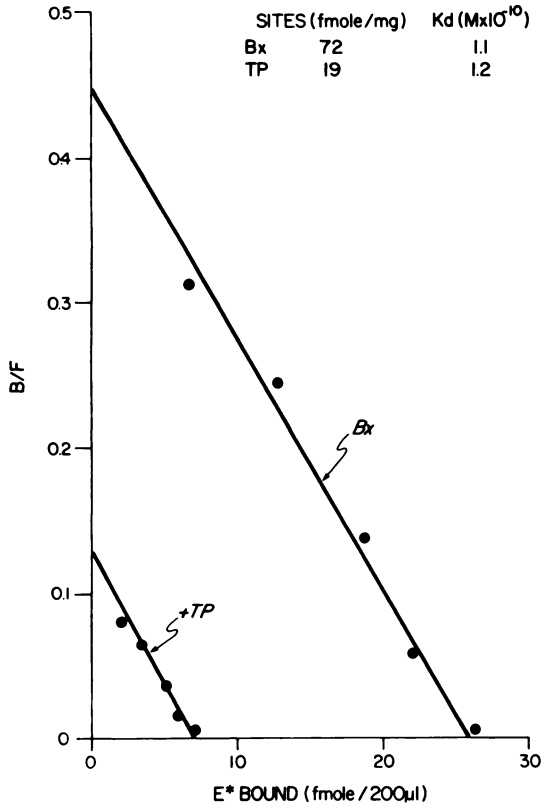

Chart 3. Typical Scatchard plot of 17β-[^{3}H]estradiol binding to estrogen receptor in DMBA rat mammary tumor before and after TP treatment. ER was measured by saturation binding with the use of the protamine assay (see text). K_d, dissociation constant; *Bx*, biopsy; *TP*, 1.2 mg TP injected 2 times weekly; E*, 17 β-[^{3}H]estradiol; B/F, bound free.

was accomplished within a few hr. The net result could be cytoplasmic ER translocated to inappropriate nuclear acceptor sites, and then eliminated, leaving insufficient cytoplasmic ER to carry out estrogen-mediated events required for growth of the tumor cell.

In considering other possibilities, Vignon and Rochefort (21) reported that prolactin stimulates ER in DMBA tumors, and we have shown the same results in rat liver (3). We have recently shown a significant decrease in prolactin receptors in DMBA tumors regressing after testosterone therapy (6). It is therefore reasonable to suggest that loss of prolactin receptor may at least partially explain the reduction in cytoplasmic ER following androgen therapy. Furthermore, experiments from Meites' laboratory [Quadri *et al.* (15)] demonstrate that very large doses of prolactin are able to reverse the DMBA tumor regression caused by androgen. Since we have reported that, in rat liver, prolactin itself is capable of stimulating prolactin receptors (5), it is conceivable that very large doses of prolactin restore prolactin receptors in the regressing tumors, which in turn help to restore cytoplasmic ER sufficiently to achieve normal ER translocation and resumption of tumor growth.

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