Progesterone and Progesterone Receptors in Experimental Breast Cancer

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SUMMARY

The synthetic progestin, R5020, has been used to demonstrate a progesterone receptor (PGR) in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors which binds hormone with high affinity (Kd ~ 1 nM) and migrates at 7S and 4S on sucrose density gradients. Rats bearing growing tumors were ovariectomized-adrenalectomized at proestrus, tumors were biopsied 24 hr later, and estrogen receptor and PGR were determined by a single, saturating-dose dextran-coated charcoal assay. PGR values averaged 247 ± 27 fmoles/mg cytosol protein. When rats received injections of progesterone plus estradiol, biopsied tumors resumed growth and PGR levels were maintained. PGR was also unchanged in tumors growing on estradiol alone. Tumors regressed after withdrawal of progesterone plus estradiol or of estradiol alone, and PGR uniformly fell to basal levels (<50 fmoles/mg cytosol protein). Estrogen treatment of regressed tumors restored both tumor growth and PGR.

In sum, castration with adrenalectomy induced rapid tumor regression and loss of PGR. Estradiol administration prevented tumor regression and PGR loss or restored PGR in regressed tumors. Progesterone alone failed to sustain tumor growth despite the initial presence of PGR.

INTRODUCTION

It is now generally appreciated that human breast tumors containing ER often regress after endocrine therapy whereas those tumors lacking ER usually fail to respond (16). The fact that not all ER-containing tumors respond has led to the concept that ER is a necessary but not sufficient marker of hormone dependence. Since ER is only an early step in the pathway from hormone binding to ultimate cellular response, it is possible that in endocrine-resistant, ER-positive tumors, lesions exist in later steps concerned with the action of the hormone. In that case a product of hormone action would be a better marker of endocrine responsiveness than the initial binding step. In the uterus, the synthesis of PGR is dependent upon the action of estrogen (20). We have demonstrated PGR in human breast tumors (7) and have hypothesized that the presence of this receptor in ER-positive tumors would indicate that the tumor is capable of synthesizing at least 1 end product under estrogen regulation and may therefore be endocrine responsive (8). Although the hypothesis assumes that PGR levels in breast tumors, like those in the uterus, are under estrogen control, this priming effect has never been demonstrated. In this paper we show that PGR in the DMBA-induced rat mammary tumor is under acute estrogen control and that PGR is rapidly depleted in tumors regressing after estrogen withdrawal.

MATERIALS AND METHODS

DMBA Tumor Induction and Growth

Fifty-day-old female Sprague-Dawley rats were given 20 mg DMBA in sesame oil by gastric intubation. When mammary tumors were palpable they were measured with callipers 3 times a week, and the tumor area was plotted on a growth chart. Only tumors developing less than 5 months after DMBA treatment were used in these studies, since such tumors are more likely to be hormone dependent (2). If more than 1 tumor appeared on the animal, one of them was designated the major tumor. When this tumor reached 2 sq cm, vaginal smears were obtained daily before 10 a.m. Rats showing 2 consecutive 5-day cycles with continued tumor growth were bilaterally OVD-ADX at proestrus. Half of the tumor was resected 18 to 24 hr later, and was trimmed and weighed; then, after removal of a sample for histology, it was frozen in liquid nitrogen. The skin was loosely sutured to permit wound drainage. All other tumors were removed and discarded. Tumor growth was maintained by daily s.c. injections of 17β-estradiol (1 μg/0.2 ml propylene glycol) plus progesterone (4 mg/0.2 ml propylene glycol). When tumors regained at least prebiopsy size they were entered into 1 of 4 protocols: (a) estradiol plus progesterone was continued; (b) estradiol was removed (progesterone alone); (c) progesterone was removed (estradiol alone); or (d) both hormones were removed (no treatment). If tumor growth continued for 10 to 12 days or if greater than 50% regression occurred, treatment was stopped and, 18 to 24 hr later, rats were killed and tumors frozen in liquid nitrogen for later analysis. Treated tumors and corresponding biopsied controls were assayed together.

In a 2nd study, to determine the effect of estrogen in regressing tumors, rats were OVD-ADX when their tumors reached greater than 3 sq cm. After more than 50% regression had occurred, growth was reinstated with estradiol...
alone (1 μg/day). When tumors regained presurgery size, estrogen treatment was stopped, and tumors were removed for assay 18 hr later.

Homogenization and Cytosol Preparation

All procedures were performed at 0-4°. Frozen tissue was crushed with a Thermovac tissue pulverizer (Thermovac Industries, Copiague, N. Y.). Weighed powder was placed in a 15-mℓ Corex tube, thawed to 0-4°, and homogenized in 2 volumes of buffer with three 10-sec bursts of a Polytron PT-10-ST (Brinkmann Instruments, Inc., Westburg, N. Y.). The homogenization buffer, prepared immediately before use, was 5 mM sodium phosphate, pH 7.4, at 4°, containing 1 mM thioglycolic acid and 10% glycerol. Cytosol was obtained by 50-min centrifugation at 40,000 rpm (100,000 × g). Protein concentration was estimated by absorbance (10) and later quantitated by the method of Lowry et al. (13).

Sucrose Density Gradients (PGR)

\[^{3}H\]R5020 ([6,7-\(^{3}H\)-17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; 51.4 Ci/mmmole; Roussel-UCLAF, Paris, France) was added at 2 × 10\(^{-8}\) M in 2 μℓ ethanol to 250 μℓ undiluted cytosol and incubated for 4 ℓ. Parallel samples were preincubated for 15 ℓ with 100-fold excess unlabeled R5020. Pellets were prepared from a 1-ml suspension of DCC (0.25% Norit A-0.0025% dextran in 10 mm Tris-HCl, pH 8.0, at 4°) by a 10-min centrifugation at 3200 rpm (2000 × g). The supernatant buffer was discarded, and charged cytosol was transferred onto the pellet, mixed, and incubated for 10 ℓ to adsorb unbound radioactivity. After centrifugation for 10 ℓ, a 200-μℓ aliquot of the supernatant was applied to a 5 to 20% sucrose gradient prepared in homogenization buffer. Discontinuous gradients were prepared manually at room temperature in 4.0-ml polyallomer tubes (Beckman Instruments, Inc., Palo Alto, Calif.) and allowed to diffuse at 4° overnight. \(^{14}C\)-Labeled bovine serum albumin, 1500 cpm/10 μℓ buffer, was added to each cytosol as an internal marker (21). Gradients were centrifuged in a Beckman Model SW 60 Ti rotor at 53,000 rpm for 16.3 ℓ. Fractions (0.2 ℓ) were collected from the bottom of the centrifuge tube by displacement with paraffin oil and counted in 5 ml modified Bray’s solution. The data were analyzed according to the method of Scatchard after subtraction of nonspecific binding calculated from the preparations competed with nonradioactive hormone.

Single-Saturating-Dose DCC Assay (ER and PGR)

PGR. Based on Scatchard plot data for several frozen tumors, a single-saturating-dose DCC assay using 10\(^{-8}\) M \[^{3}H\]R5020 and 10\(^{-4}\) M unlabeled R5020 was used for receptor level determinations (Table 1). Preliminary experiments (data not shown) showed that the amount of specifically bound steroid was linearly related to protein concentrations between 0.5 and 10 mg/ml and that binding reached a maximum by 1 ℓ incubation at 4° and remained stable for 18 ℓ. A 4-ℓ incubation using cytosol at 1.2 to 2.0 mg/ml protein was routinely used.

ER. The single-dose ER assay is a modification of the procedure described by Leung et al. (11), using a 8 × 10\(^{-8}\) M final concentration of 17β-[\(^{3}H\)]estradiol [2,4,6,7-\(^{3}H\)]estradiol 1,3,5(10)-triene-3,17β-diol; 100 Ci/mmmole; New England Nuclear, Boston, Mass.) and 100-fold excess unlabeled diethylstilbestrol (α,α-diethylstilbestrol) as competitor.

RESULTS

Single-Saturating-Dose Assay and Receptor Content of Mammary Tumors

The single-saturating-dose assay for ER has excellent correlation with values obtained from Scatchard plots and sucrose density gradients (17). Tumor ER levels greater than 3 fmoles/mg cytosol protein by single-saturating-dose assay have saturable 8 S and 4 S peaks of more than 3 fmoles/mg on sucrose gradients.

In fresh DMBA-induced tumors, PGR is often seen in both 7 S and 4 S peaks on sucrose gradients. In frozen DMBA-induced tumors, PGR is seen primarily in the 4 S region with a 6 S shoulder (Chart 1). Although some tumors do not have either 7 S peaks or 6 S shoulders, all tumors have a low basal level of PGR by DCC-Scatchard assay (Chart 2). We find that tumors containing more than 50 fmoles PGR per mg cytosol protein usually have saturable 6 to 7 S binding, whereas tumors containing less than 50 fmoles PGR per mg cytosol protein have only a small amount of saturable binding confined to the 4 S region of the sucrose gradient. The contrast in PGR between growing and regressing tumors also suggests that a basal level of PGR is present in all

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Table 1 Comparison of PGR concentration in sucrose density gradient, Scatchard, and single-saturating-dose assays (fmoles/mg cytosol protein) in 4 frozen tumors
Progesterone Receptors in Breast Cancer

sied 18 to 24 hr later and then placed on estradiol plus progesterone to stimulate tumor growth (Chart 3). Most biopsied tumors healed well, attained at least prebiopsy size, and were used when growth was clearly established in about 21 days (range, 5 to 58 days). At this point treatments were changed to either estradiol alone, which usually continued to stimulate tumor growth, progesterone alone, or no treatment. In the latter 2 groups 22 of 23 tumors promptly regressed (greater than 50% regression in 9.6 ± 0.9 days).

Hormone Treatment and Receptor Levels

Estrogen Plus Progesterone. Both pre- and posttreatment tumors had a wide range of values for both ER and PGR, although the average values were similar (Chart 4). We conclude that the surgery and substitution with exogenous hormone treatment had little consistent effect on receptor levels.

Hormone Treatment and Growth Response of Biopsied Tumors

All rats were OVD-ADX at proestrus. Tumors were biop-
**Estrogen Alone.** When tumors growing on estradiol and progesterone were switched to estradiol alone, 5 of 7 tumors continued to grow without significant changes in either ER or PGR (Chart 5). Two of 7 tumors regressed after progesterone withdrawal and were associated with significant PGR loss. We might speculate that these tumors were dependent upon progesterone for growth and that the fall in PGR was a result of tumor regression. Otherwise, it would be difficult to explain the PGR loss in the presence of adequate estradiol and ER in these tumors.

**Progesterone Alone.** When estradiol was withdrawn from tumors growing on estradiol and progesterone, 11 of 13 tumors regressed rapidly. This was accompanied by a modest but significant fall in ER and a precipitous fall in PGR (Chart 6). Despite estrogen withdrawal, 2 tumors failed to regress and may have either been autonomous or progesterone dependent. These were the only tumors in which ER levels increased. It has been reported that progesterone can increase the uptake of [3H]estradiol in DMBA-induced tumors (19).

**No Therapy.** When both hormones were withdrawn from tumors growing on estradiol and progesterone, rapid tumor regression occurred accompanied by decreases in ER and precipitous loss of PGR (Chart 7). We have shown, therefore, that in 22 of 24 tumors regressing after estrogen withdrawal (Charts 6 and 7), PGR rapidly falls to baseline levels (<50 fmoles/mg cytosol protein). It is unlikely that all of the PGR loss is the result of cell death, since ER is preserved, although slightly reduced over the same time interval. Mammary tumor PGR seems to be much more sensitive to estrogen withdrawal than uterine PGR, which takes up to 3 weeks to fall to baseline levels (unpublished observation). We have observed tumor PGR to fall to baseline as early as 5 days after estrogen withdrawal.

**Estrogen Rescue of Regressing Tumors.** Since PGR fell rapidly after estradiol withdrawal, the next critical question was whether the tumor PGR could be restored with estradiol injections. OVD-ADX rats bearing regressing tumors
these properties has been demonstrated when progesterins with high affinity (Kd approximately 1 nM) and migrates at least in part at 7 S on sucrose density gradients. Neither of this question since over 95% of our induced tumors were found ER in 39 of 40 biopsy specimens, we cannot resolve content, the mean ER level was the same at the end of each treatment irrespective of whether the tumor was actively growing or regressing. This is in contrast to the mean PGR level which was high in the presence of estrogen and low in the absence of estrogen.

DISCUSSION

The synthetic progesterin R5020 has been used to demonstrate a PGR in DMBA mammary tumors (1). The receptor resembles PGR of other tissues in that it binds hormone with high affinity (Kd approximately 1 nM) and migrates at least in part at 7 S on sucrose density gradients. Neither of these properties has been demonstrated when progesterone is the ligand (5).

The presence of ER in DMBA-induced tumors is well documented (4, 6, 12, 18, 23). Although some investigators have suggested that estrogen-binding capacity is correlated with hormone dependence, others have shown that essentially all tumors have measurable ER (4, 6). Although we found ER in 39 of 40 biopsy specimens, we cannot resolve this question since over 95% of our induced tumors were also hormone dependent.

ER levels in DMBA-induced tumors have been shown to increase with exogenous prolactin treatment (24). We have seen no enhancement of ER in estradiol-treated groups, suggesting that the endogenous prolactin stimulation achieved by daily 1-μg estradiol injections is insufficient to reproduce the effect of exogenous prolactin.

Our studies show that in mammary tumors, as in the uterus (20), estrogen exerts acute control over PGR. Since estradiol stimulates prolactin secretion (4), we have not ruled out the possibility that endogenous prolactin may be stimulating PGR. Although we are testing this possibility in current studies, we believe that prolactin is not involved, since estrogen alone but not prolactin can stimulate synthesis of PGR in the uterus of triple-operated rats (OVD-ADX, hypophysectomized) (unpublished observations).

Although progesterone clearly enhances induction of DMBA tumors (9), its role in the growth of established tumors is unclear, as is the mechanism by which progestins induce tumor regression (15, 22). Our data show that in the absence of estradiol, physiological doses of progesterone do not sustain growth of established tumors despite the initial presence of PGR. It may be that cytoplasmic PGR is depleted by progesterone injection and no further PGR synthesis occurs in the absence of estradiol. This would render the cell refractory to further stimulation by progesterone. The basal PGR level seen in all tumors appears to be inadequate in mediating a potential progesterone effect on tumor growth. Whether it is an effective mediator of other progesterone functions in mammary tissue remains to be seen.

In considering the role of progesterone and PGR in mammary tumors, it is important to distinguish between 2 points: (a) the direct role of progesterone in hormone-dependent growth and regression; (b) its use as a marker of estrogen action and ER integrity. We have been measuring PGR in human breast cancer (8) for the purpose of demonstrating an intact estrogen response system. In rat tumors we have now shown that a relationship exists between the presence of estradiol and the presence of PGR. This is not to say, however, that estrogen-responsive tumor growth and estrogen-induced PGR are inexorably linked. At least 2 tumors grew in the absence of estradiol so that their growth may be considered estradiol autonomous, whereas their PGR declined and could therefore be considered estrogen dependent. This suggests some dissociation between estrogen-controlled tumor growth and estrogen-controlled PGR synthesis. Similarly, from the persistent basal level of PGR seen in estradiol-deprived tumors and in the 2 tumors that failed to respond to estradiol, we must conclude that at least some PGR appears to be estrogen independent.

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