Prolactin Receptors and Androgen-induced Regression of 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinoma

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INTRODUCTION

It is well established that prolactin is important for growth of experimental mammary carcinomas. Reduced or circulating prolactin with drugs (1, 17) or by pituitary removal (19) results in tumor regression, whereas increasing circulating prolactin enhances tumor growth (3, 9, 16, 18–20, 23). Although it has been proposed that prolactin alone is responsible for hormone-dependent tumor growth in DMBA-induced mammary tumors (18, 19), recent studies indicate that estrogen may be required for prolactin to exert its effects (3, 10, 11, 23).

Previous work in this laboratory has shown that prolactin receptors are present in an ovarian-dependent subline of the prolactin-dependent MTW9 mammary tumor. In an autonomous subline from the same parent tumor, there is a 7-fold reduction in these receptors (6). The results suggested that a relationship might exist between the presence of prolactin receptors and hormone-dependent growth characteristics. Similarly, Kelley et al. (9) have found that the number of prolactin receptors in DMBA-induced mammary tumors correlates with the growth response to injected prolactin.

SUMMARY

Prolactin reverses the inhibitory effects of pharmacological doses of androgen on 7,12-dimethylbenz(a)anthracene-induced mammary tumor growth (Quadri, S. K., Kledzik, G. S., and Meites, J. J. Natl. Cancer Inst., 52: 875–878, 1974). To determine whether this effect is due to an alteration in the ability of the tumor cell to bind prolactin, we have quantitated prolactin receptors in androgen-responsive and nonresponsive tumors. Prolactin receptors were measured with 125I-labeled ovine prolactin in a subcellular fraction which reproducibly contained 60 to 80% of the total receptor present in tumor homogenates. Prolactin binding was reversible, reached a steady state in 9 hr, and was completed by excess unlabeled prolactin. Prolactin bound to its receptor with a $K_d$ of approximately 1 x 10^{-10} M. Growing tumors were biopsied, and rats bearing regrown tumors were given injections of 4 mg testosterone propionate twice a week. Prolactin receptors were reduced in most of the tumors, which regressed after testosterone treatment by an average of 63% compared to the pretreatment biopsy specimens. Nonresponsive tumors and vehicle-injected controls showed no significant alterations in receptor content. This reduction of prolactin receptors is probably insufficient to account for androgen-induced mammary tumor regression.

MATERIALS AND METHODS

Mammary tumors were induced in 50- to 55-day-old virgin female Sprague-Dawley rats (Holtzman, Co., Madison, Wis.) by administration of 20 mg DMBA (Sigma Chemical Co., St. Louis, Mo.) in peanut oil by gavage. Two to 4 months later, tumors appeared, and their growth was charted by measurement of tumor size with calipers. When a tumor reached 2 to 4 sq cm (length x width), less than one-half the tumor mass was biopsied, and all other tumors were excised and discarded. All biopsies were performed when rats were in diestrus as determined by daily vaginal smears. The tumor tissue was freed of debris and weighed, and a histological section was taken before freezing in liquid N2 and storing in a Revco freezer at −70°. Regrowth of the remaining tumor was charted, and, when it reached the prebiopsy size, the rat received an injection of 4 mg TP in 0.2 ml sesame oil on Monday and Friday of each week (controls received sesame oil alone). Tumors were then classified as responders (regression) or nonresponders (continued growth). Responders were sacrificed the day after the last injection when tumors showed 50% regression. In the case of nonresponders and controls, animals were sacrificed when they...
had received injections for at least the number of days that caused 50% regression in responsive tumors. These tumors were frozen and handled in the same manner as biopsies. Only tumors that were clear adenocarcinomas were assayed.

**Prolactin Receptor Assay.** To measure prolactin receptors in tumors, we have developed a method to obtain most of these receptors in a single particle fraction essentially free of nuclei and debris. Frozen tumor tissue was pulverized with a Thermovac tissue pulverizer, and a 0.5-g portion was suspended in 4.5 ml of 10 mM sodium phosphate-0.15 M NaCl, pH 7.0, at 4°C. Homogenization was accomplished by three 30-sec bursts with a Polytron PT-10-ST at a setting of 3.5. The homogenate was poured through organza cloth, and the tube and mesh were rinsed with 1 ml 0.5 mM CaCl₂. One ml of the homogenate was removed, brought to 40 ml with 0.5 mM CaCl₂, and centrifuged at 100,000 × g for 50 min. The pellet was resuspended in 3.0 ml Buffer A (25 mM sodium phosphate, 10 mM MgCl₂, 0.1% bovine serum albumin, and gentamicin, 10 μg/ml, pH 7.0). This fraction (designated fraction H) was used to determine total homogenate binding from which recovery in the PN fraction was calculated (see below). The remaining homogenate was brought to 40 ml with 0.5 mM CaCl₂, stirred occasionally for 5 to 10 min, and then centrifuged at 150 × g for 20 min to remove nuclei and debris. The 150 × g supernatant was centrifuged at 100,000 × g for 50 min, and the pellet (designated the PN fraction) was suspended in 6.0 ml Buffer A.

The receptor assay was run in triplicate in 12- x 75-mm polystyrene tubes previously washed in Buffer A. One-tenth ml of the PN fraction was mixed with 0.1 ml Buffer A containing ~30,000 cpm ¹²⁵I-labeled ovine prolactin with 0, 0.25, 0.5, 1, 2, 4, or 256 ng unlabeled ovine prolactin (NIHP-S-10). The H fraction was incubated with ~30,000 cpm ¹²⁵I-labeled ovine prolactin ± 256 ng unlabeled prolactin. Tubes were incubated at 22°C for 16 hr with shaking. All tubes were then counted for total cpm, and then 2.5 ml ice-cold Buffer A were added, and the particles were pelleted at 20,000 × g for 10 min at 4°C. After washing with 10 mM sodium phosphate, pH 7.0, the pellets were dissolved in 0.5 ml 0.1 N NaOH and counted. Protein was determined directly on the dissolved pellets by the method of Lowry et al. (12). Scatchard analysis (22) was performed on the binding data with correction for nonspecific binding. The fractional recovery of prolactin receptors in the PN fraction for each tumor was taken as the ratio of total specific prolactin binding in the PN and the H fraction incubated under identical conditions (~30,000 cpm ¹²⁵I-labeled ovine prolactin ± excess unlabeled prolactin for 16 hr at 22°C with the same amount of protein from each fraction). Total tumor prolactin receptor was calculated by the formula:

\[
\text{Receptor in PN fraction}\left(\frac{\text{fmol}}{\text{mg protein}}\right) \times \left(\frac{\text{total PN fraction protein (mg)}}{\text{Fractional recovery}}\right) \times \frac{1}{\text{H fraction protein (mg)}} = \text{total tumor receptor}\left(\frac{\text{fmol}}{\text{mg protein}}\right).
\]

**Results**

**Receptor Assay.** Binding of ¹²⁵I-labeled prolactin to the PN fraction was linear with protein concentration from 40 to 160 μg protein (Chart 2). Nonspecific binding increased proportionately with protein concentration and was <10% of the total prolactin bound over the entire range tested.

A steady state in specific prolactin binding to the PN fraction was achieved by 9 hr of incubation at 22°C; this level remained constant until 22 hr (Chart 3). Other experiments revealed that, beyond 24 hr, total prolactin binding decreased and nonspecific binding increased (not shown). Prolactin binding in the PN fraction was at least partially

**Prolactin Iodination.** ¹²⁵I-labeled ovine prolactin was prepared by lactoperoxidase iodination separated from free ¹²⁵I on Sephadex G-25F and purified by DEAE-cellulose chromatography as previously described (4). Specific activity ranged from 55 to 65 μCi/μg assuming 100% recovery of prolactin from the Sephadex column. The binding characteristics of the ¹²⁵I-labeled prolactin compared to those of unlabeled ovine prolactin in the PN fraction were determined as follows. PN particles were incubated to a steady state in the presence of increasing concentrations of ¹²⁵I-labeled prolactin or with a fixed amount of ¹²⁵I-labeled prolactin and increased increments of unlabeled prolactin. After correction for nonspecific binding at each dose, Scatchard analysis of the binding data resulted in an essentially straight line plot in both cases (Chart 1). Since both the slopes of the lines and the intercepts were identical, our ¹²⁵I-labeled prolactin was binding to receptor in a manner that was identical to that of unlabeled hormone. This also indicates that the calculation of the specific activity of the labeled prolactin was correct. Since much more label was consumed by using increasing amounts of ¹²⁵I-labeled prolactin, we used the fixed concentration of ¹²⁵I-labeled prolactin with increasing unlabeled prolactin for all our assays.

**Chart 1.** Receptor binding to ¹²⁵I-labeled or unlabeled ovine prolactin. The PN fraction of a DMBA tumor was incubated with 30,000 cpm ¹²⁵I-labeled prolactin and increasing concentrations of unlabeled prolactin (A) or with increasing quantities of ¹²⁵I-labeled prolactin (B). After 16 hr of incubation at 22°C, the particles were pelleted, washed, and counted as described in "Materials and Methods." Binding was corrected for nonspecific binding at each dose, and the data were plotted according to the method of Scatchard (22). Each point is the mean of triplicate samples which did not vary by more than 5% from each other.
reversible; more than 40% of the bound $^{125}$I-labeled prolactin could be displaced within 12 hr after dilution of the labeled PN fraction and addition of excess unlabeled hormone (Chart 3).

**Effect of TP on Mammary Tumor Growth.** Chart 4A shows a representative plot of tumor growth, from appearance of the tumor to regrowth after biopsy and regression following TP treatment. About 80% of tumors responded in this fashion. Regression to 50% of the peak tumor size averaged 25 days, with a range of 10 to 60 days. A few tumors did not regress with TP administration (nonresponders) (Chart 4B), and tumors given vehicle alone continued to grow (Chart 4C).

**Effect of TP on Prolactin Receptors.** Prolactin binding kinetics of a tumor that regressed after TP administration (Chart 5) shows that the receptor sites were reduced in the regressed tumor compared to the biopsy. The dissociation constant ($K_d$) remained unchanged. Chart 6 shows total prolactin receptor content in all tumors before and after treatment with TP or vehicle. Receptor content in untreated tumors (biopsies) varied over a 10-fold range (60 to 650 fmoles/mg protein). In most but not all regressors there was a reduction in prolactin receptor sites compared to the biopsy of the same tumor. The average reduction in prolactin receptors as a result of TP-induced regression was 63%, while receptor content of nonresponders and controls was relatively constant between the biopsy and treated groups (Table 1).

**DISCUSSION**

Endocrine-induced regression of experimental mammary tumors can be accomplished by several means, including drugs that reduce circulating prolactin, endocrine ablation, antiestrogens, and pharmacological doses of either andro-
mone-dependent growth characteristics (6), so that eliminating these hormones directly or indirectly results in tumor regression.

With respect to the mechanism of tumor regression as a consequence of androgen administration, the role of receptors is much less clear. The only data come from the report of Quadri et al. (20). High doses of androgen reduced both the number and size of DMBA-induced mammary tumors, and this effect was prevented by coadministration of prolactin. The authors proposed that since androgen did not reduce circulating prolactin, inhibition of tumor growth was the result of androgen interference with prolactin action at the tumor cell. Our finding that prolactin receptors were substantially reduced in many tumors regressing in response to TP tends to support this hypothesis; however, a number of other observations suggest that this may not be the sole mechanism.

First, not all tumors that regressed showed a reduction in prolactin receptors. In these tumors one might reason that some receptors in biopsies could have been occupied by endogenous prolactin, reducing the apparent number of binding sites so that a true decrease in receptors from the biopsy to the regressed tumor would not be seen. All biopsies, however, were taken in diestrus when circulating prolactin is low.

Second, it is possible that not all the tumors that regressed following TP administration were solely dependent on prolactin for growth. Recent results from Leung and Sasaki (10) have shown that prolactin alone is only weakly stimulatory for tumors that have regressed following ovariectomy and adrenalectomy. Coadministration of 0.01 μg estradiol, which by itself does not stimulate tumors, resulted in a reactivation of tumor growth (10). In these tumors, regression might be mediated by another mechanism, perhaps by an androgen-induced decrease in cytoplasmic estrogen receptors as in the rat uterus (21).

Third, although prolactin receptors were not completely abolished in responsive tumors, they were significantly reduced by an average of 63%. One might hypothesize that a certain level of biochemical signal in response to prolactin may be necessary to support tumor growth. If either prolactin receptors or the prolactin level were reduced so that the

### Table 1

<table>
<thead>
<tr>
<th>Tumor group</th>
<th>Receptor (fmoles/mg protein)</th>
<th>$P^*$</th>
<th>$N$</th>
<th>$K_d$ ($\times 10^{16}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bx(R)</td>
<td>260 ± 46</td>
<td>&lt;0.01</td>
<td>12</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>R</td>
<td>95 ± 16</td>
<td></td>
<td>12</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Bx(NR)</td>
<td>156 ± 18</td>
<td>&gt;0.6</td>
<td>4</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>NR</td>
<td>138 ± 23</td>
<td></td>
<td>4</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Bx(C)</td>
<td>201 ± 30</td>
<td>&gt;0.2</td>
<td>11</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>C</td>
<td>173 ± 29</td>
<td></td>
<td>11</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

$p$ for changes from biopsy values to corresponding pretreatment values (paired data). $P$ values for Bx(R) vs Bx(NR), 0.2; Bx(NR) vs Bx(C), >0.5; Bx(R) vs (C), >0.2. Total prolactin receptors were calculated as described in "Materials and Methods."

The abbreviations used are: Bx, biopsy; R, responsive; NR, nonresponsive; C, vehicle-injected control.

Each value is the mean ± S.E. for $n$ tumors.
critical level of signal was not obtained, regression would occur. Tumor response to prolactin has been shown to be dose related: Nagasawa and Yanai demonstrated that injection of 2.5 mg prolactin twice a day stimulated DMBA-induced tumor growth, whereas 1.25 mg were essentially ineffective (18), and the transplantable MTW9 rat mammary carcinoma will only grow if circulating prolactin is raised to supraphysiological levels by coimplantation of a prolactin-secreting pituitary tumor (13). With respect to tumor receptor content, our own results may seem to support this hypothesis, since prolactin injection could overcome an inadequate signal by increasing receptor occupancy and perhaps also receptor number, as in rat liver (5). However, all of our tumors grew on physiological levels of prolactin regardless of their prolactin receptor content, so that, unless each tumor had a unique level of signal to support tumor growth, such a hypothesis could not adequately explain our findings.

Finally, we should be cautious not to focus only on prolactin receptor modulation in controlling breast tumor growth and regression. Other mechanisms are also possible. Regression of DMBA-induced mammary tumors following ovariectomy is accompanied by a reduction in estrogen receptors. Prolactin reverses regression and increases estrogen receptors in these tumors (24, 25). Similarly, prolactin causes a partial restoration of liver estrogen (2) and gastrin receptors alone is probably insufficient to account for androgen-induced mammary tumor regression. Such a hypothesis could not adequately explain our findings.

A Comment on Methods to Determine Prolactin Binding in DMBA-induced Tumors. When this study was initiated, we anticipated that receptor recovery in subcellular fractions might vary considerably due to the variety of pathological types in these adenocarcinomas. Regression caused by TP, as well, might be expected to alter receptor recovery. Because such variations could markedly affect interpretation of our results, we determined total prolactin receptor content in tumors by Scatchard analysis of binding in the PN fraction and corrected these values for loss due to fractionation. We have found that recovery of receptor in the PN fraction does not vary considerably between tumors. Recovery of receptor in the PN fraction was unaltered regardless of treatment or response: 66 ± 2.6% for all biopsies; 68 ± 2.8% for regressors, 73 ± 6.3% for nonregressors, and 63 ± 2.5% for vehicle-injected controls. Thus, evaluating our data based on Scatchard analysis of prolactin binding in the PN fraction alone does not change the interpretation of our results (Table 2). In addition, single-point assays on the H fraction under nonsaturating conditions also show a reduction in prolactin binding in the TP-treated tumors. However, this type of assay does not indicate total receptor content and receptor binding affinity.

Although somewhat more laborious, we feel that measuring total prolactin receptor content as described here is superior to using 1-point assays or performing Scatchard analysis on subcellular fractions alone because total tumor receptor quantity and a Kd are obtained. The method should potentially be useful in determining relatively small changes in receptor, since saturating conditions are used and a Kd is also obtained. Variations in receptor recovery between different target tissues and the potential effects of various treatments on receptor distribution during fractionation can also be evaluated.

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REFERENCES

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