Prolactin and Progesterone Receptors in Pregnancy-Dependent Mammary Tumors in GR/A Mice (43158)

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Abstract. **In this study, cellular prolactin receptors and cytosolic progesterone receptors were examined and compared in pregnancy-dependent mammary tumors (PDMT) and in normal mammary glands of pregnant GR/A mice. PDMT and normal mammary glands were examined in the same animal, thus assuring an identical hormonal environment. The PDMT cells had a larger capacity to bind prolactin or the synthetic progesterone, R5020, than did the normal mammary gland. While the dissociation constant (K_d) value for prolactin binding to normal mammary epithelial cells was similar to that of PDMT cells, PDMT cells had 2.2 times more prolactin receptors than the normal cells. Progesterone binding activity was detected only in PDMT, but not in the normal mammary cells. The receptor concentration and the K_d value for progesterone binding of PDMT were 606 fmol/mg protein and 3.53 n*M*, respectively. It appears, therefore, that normal regulation of these receptors may be altered within the PDMT cells. The increased growth responsiveness of PDMT to the hormones of pregnancy, especially prolactin, progesterone, and placental lactogen, may be a function of a sharp increase in the level of cellular receptors for these mammotropic hormones.** [P.S.E.B.M. 1990, Vol 195]

Pregnancy-dependent mammary tumors (PDMT) of GR/A mice first appear after midpregnancy, have maximal size at the end of pregnancy, and disappear quickly after parturition whether or not the mice undergo lactation (1). Approximately 30% of PDMT reappear after retirement as malignant mammary tumors and PDMT are considered by some investigators to be preneoplastic (1). Like normal mammary glands, the growth of PDMT is progesterone responsive (2, 3). The growth stimulatory effect of progesterone is enhanced by placental lactogen (2), a peptide that is similar to prolactin in effecting mammary gland growth (4). Placental lactogen can bind to the same receptor for prolactin (5). However, the pattern of growth response of PDMT and the normal mammary gland to these hormones is obviously different as PDMT cells quickly evolve into palpable tumors during the relatively short time span of pregnancy (2, 3). In this article, prolactin and progesterone receptors of PDMT and normal mammary gland were examined and compared for the purpose of clarifying the very apparent growth differential of these cells that occurs during the physiologic state of pregnancy.

Materials and Methods

Animals and Treatments. GR/A mice maintained in our laboratory by strict brother × sister mating were mated with males at 45–50 days of age. Pregnant animals were housed individually and placed again with males only near parturition to induce concomitant pregnancy and lactation. Litter size was adjusted to five or six on the day of parturition (Day 0 of lactation) and allowed to nurse normally. Only mice that became pregnant consecutively and had PDMT at the second pregnancy were used in this study. Incidence of PDMT

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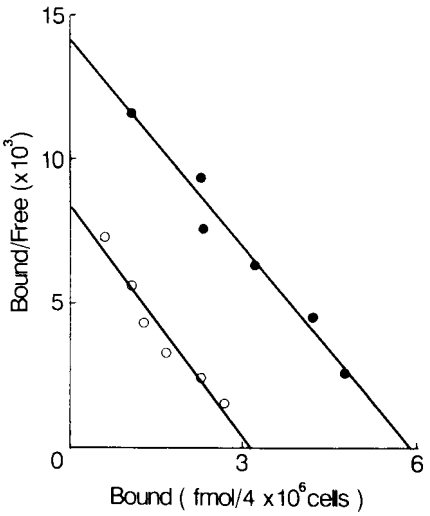

Figure 1. Scatchard analysis of prolactin binding in normal mammary gland (○) and in PDMT (●). The dissociated mammary epithelial cells (4×10^6 cells/tube) were incubated with 100,000 cpm of ^{125}I-ovine prolactin in the presence of 0, 2.5, 5, 10, 20, and 40 ng or 5 μg of unlabeled prolactin. Bound and bound/free values were plotted according to Scatchard (9). Results of other two experiments were identical to those in this figure.

in this strain is approximately 10 and 60% at the first and the second pregnancies, respectively (6), which was the reason why we used mice at the second pregnancy. On Days 19 and 20 of the second pregnancy, all mice were killed by decapitation under light ether anesthesia, and PDMT and normal mammary glands lacking PDMT were removed and stored at −80°C for prolactin and progesterone receptor analysis.

Prolactin Receptor Assay Using Dissociated Mammary Epithelial Cells. A precise protocol for iodination of prolactin, digestion of mammary tissue with collagenase, and *in vitro* prolactin binding assay has previously been described in detail (7). The specific radioactivity of ovine prolactin (NIADDK-S-P17, 30 IU/mg) iodinated with ^{125}I by lactoperoxidase was 2.6 MBq/μg. Mammary tissues combined from three mice were finely chopped and incubated in 30 ml of Medium 199 containing 30 mg of collagenase, 3 mg of DNAse, and 300 mg of bovine serum albumin for 60 min at 37°C in a shaking water bath. At the end of the incubation, the mixture was filtered through 150-μm nylon mesh. The filtered cells were centrifuged at 80*g* for 1 min to obtain aggregated epithelial cells. Finally, the cell suspension was adjusted to 1×10^7 cells/ml in medium 199-0.25% bovine serum albumin (binding buffer). The cells (4×10^6) were incubated for 120 min at room temperature with ^{125}I-prolactin (100,000 cpm) in the presence of various concentrations of unlabeled prolactin in a total reaction volume of 0.5 ml. The amount of total and nonspecific binding of ^{125}I-prolactin to the cells was estimated in the absence or presence of 5 μg of unlabeled prolactin, respectively. After incubation, mammary cells were washed twice with 1 ml of binding buffer. The radioactivity bound to the cells was measured in an autogamma counter with counting efficiency of 60%. The experiments were performed in triplicate and each determination was repeated three times. Prolactin binds to its receptor in a 1:1 ratio (8) and the molecular weight of ovine prolactin is 23,000. The number and the dissociation constant (K_d) of prolactin receptors were estimated according to Scatchard (9).

Measurement of Cytosolic Progesterone Receptor. Mammary tissues were weighed and homogenized in 10 volumes (v/w) of TEDMG buffer (10 m*M* Tris-HCl, 1 m*M* disodium EDTA, 1 m*M* dithiothreitol, 10 m*M* sodium molybdate, and 10% (v/v) glycerol, pH 7.4) with a Physcotron (NITI-ON, Tokyo, Japan). All subsequent procedures were carried out at 0–4°C. The tissue homogenate was centrifuged at 800*g* for 15 min. The surface lipid was discarded and the low-speed supernatant was spun at 105,000*g* for 1 hr by a Hitachi SCP 85H model ultracentrifuger (Hitachi Koki Co., Tokyo, Japan) to obtain cytosol fraction. Protein concentration of the cytosol was determined by Coomassie blue staining of the Bio-Rad protein assay kit with bovine serum albumin as a standard (10).

The cytosol (0.25 ml) was incubated in duplicate with either increasing concentrations (0.78-25 n*M*) of [^{3}H]R5020 (specific activity = 2.59–3.22 TBq/mmol; New England Nuclear, Boston, MA) or [^{3}H]R5020 plus a 100-fold molar excess of unlabeled R5020 (78–2,500 n*M*) for 18 hr at 0°C. At the end of the incubation, an equal volume of 60% (v/v) hydroxylapatite (Bio-Gel HAP; Bio-Rad, Richmond, CA) suspension was added

Table I. Specific Binding, Dissociation Constant, and Binding Site for Prolactin in Normal Mammary Gland and PDMT

	No. of samples	Specific binding (cpm/4 × 10^6 cells)	Dissociation constant (K_d; n*M*)	Binding site (site/cell)
Normal glands	3	723 ± 94[a]	0.76 ± 0.05	402 ± 35
PDMT	3	1412 ± 110	0.88 ± 0.06	899 ± 83
P		<0.02	>0.05	<0.02

[a] Mean ± SE.

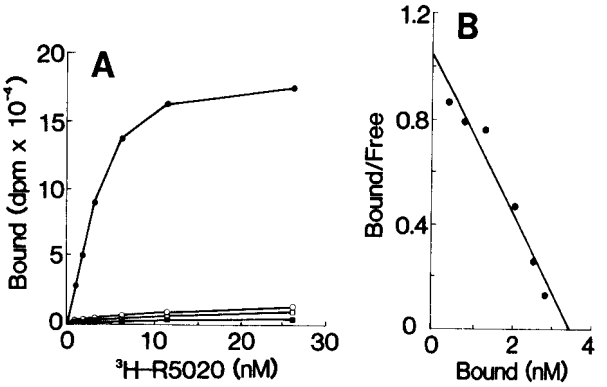

Figure 2. Representative data of saturation and Scatchard analysis. (A) Saturation analysis of [^{3}H]R5020 binding in the cytosol of the normal mammary glands (□, ■) or PDMT (○, ●). Aliquots (0.25 ml; 1.3 mg of protein) of cytosol were incubated with increasing concentrations (0.78–25 n*M*) of [^{3}H]R5020 (total binding) and in parallel with the same concentration of the radioactive steroid plus a 100-fold excess of radioinert R5020 (nonspecific binding) for 18 hr at 0°C. Specific binding (●, ■) was obtained by subtracting nonspecific binding (○, □) from total binding. (B) Scatchard analysis of the binding of R5020 to the cytosolic receptor of PDMT. A straight line was obtained by the analysis of the data. The K_d value obtained from the slope of the regression line was 3.24 n*M* and the maximal concentration of the binding site was 656 fmol/mg protein.

to each tube and the adsorption continued for 15 min with occasional vortexing according to the method of Watson and Clark (11). Two milliliters of cold TEDMG buffer were then added to the mixture and the tubes were centrifuged at 800*g* for 5 min. The pellets were washed three times with 2 ml of TEDMG buffer and extracted with 1 ml of absolute ethanol for 30 min at room temperature. Three milliliters of scintillation fluid (Atomlight; New England Nuclear) were added to each extract and the radioactivity was measured. Specific binding was obtained by subtracting nonspecific binding from total binding and plotted according to the method of Scatchard (9).

Results

Prolactin Receptor. After digestion of the tissues with collagenase, the dissociated epithelial cells in PDMT and in normal mammary glands were isolated by low-speed centrifugation. The final cell yields combined from three animals were 1.9–2.2 × 10^8 cells in PDMT and 0.97–1.2 × 10^8 cells in normal mammary glands (n = 3). The size of cells derived from normal mammary gland was 2- to 3-fold greater than that derived from PDMT. Cell size was estimated by a phase contrast microscope. Furthermore, DNA contents per mg dry fat-free tissue were 25.3 ± 2.0 μg in normal glands and 53.9 ± 2.4 μg in PDMT.

The amount of specific binding of prolactin in the PDMT cells was significantly greater than that in the normal cells.

Scatchard analysis was carried out using the dissociated epithelial cells of PDMT as well as of normal mammary glands in order to characterize the receptor further. As shown in Figure 1, both Scatchard plot lines were parallel to each other. This indicates that the K_d value for the binding of prolactin did not differ between normal mammary gland and PDMT cells. However, the plot lines were clearly shifted. The cells obtained from PDMT contained more prolactin binding sites than those obtained from normal glands. Results obtained from three determinations are summarized in Table I. The PDMT cells had approximately 2.2-fold more prolactin binding sites than did normal cells. Over 90% of the bound prolactin was dissociated from the cells in less than 5 min by exposure to pH 3.0 (0.1 *M* ammonium acetate). This suggests that most receptors analyzed here are located on the cell surface. Since the surface area of the PDMT cells was much smaller than that of the normal cells, the receptor concentration in the PDMT cells would be considerably greater than that of the normal cells.

Progesterone Receptor. Saturation analysis experiments were performed by incubating aliquots of cytosolic fraction with increasing concentrations of [^{3}H]R5020 (0.78–25 n*M*). Figure 2A shows that the specific binding of [^{3}H]R5020 in cytosolic fraction obtained from PDMT saturated between 10 and 20 n*M*. Scatchard analysis of the PDMT data in Figure 2A revealed a single class of high affinity binding sites with a K_d value of 3.24 n*M* and the maximum number of binding

Table II. Dissociation Constant and Binding Site for R5020 in the Cytosol from Normal Mammary Gland and PDMT

	No. of samples	Dissociation constant (K_d; n*M*)	Binding site	(fmol/mg protein)
Normal glands	4	ND[a]	ND	7 ± 4[b]
PDMT	4	3.53 ± 0.36[c]	606 ± 155[d]	475 ± 112[b]
P				<0.01

[a] Not detectable.
[b] Evaluated by single saturating dose assay with 25 n*M* [^{3}H]R5020 in the presence or absence of a 100-fold excess of radioinert R5020.
[c] Mean ± SE.
[d] Determined from the *x*-intercept of the Scatchard plot.

sites of 656 fmol/mg protein (Fig. 2B). In contrast, Scatchard plot of normal mammary gland data was not obtained. Thus, to compare the number of progesterone receptors between the two tissues, the progesterone binding site was evaluated from the amount of [^{3}H] R5020 specifically bound when incubated under equilibrium conditions with 25 n*M* [^{3}H]R5020 in the presence or absence of a 100-fold excess of radioinert R5020 (a single saturating dose assay). Table II shows that the PDMT contain about 70-fold more progesterone binding sites than did normal mammary glands.

Discussion

In this study, PDMT and normal mammary glands were isolated from the same animals and, therefore, both tissues were under the identical hormonal environment. The results of this study show that levels of specific cellular binding sites for prolactin were significantly higher in PDMT than in normal mammary glands. This indicates that PDMT cells have much higher susceptibility to prolactin than the normal mammary gland cells and would explain an increased growth response of PDMT to prolactin and placental lactogen at the end of pregnancy. It was also reported that the prolactin receptor level was 3-fold higher in prolactin-dependent rat mammary tumors induced by dimethylbenz[*a*] anthracene than in normal lactating glands (12).

The normal mammary glands did not contain a detectable amount of progesterone receptor in the cytosolic fraction. In contrast, a large quantity of progesterone receptor was detected in PDMT, supporting the increased growth susceptibility of PDMT to progesterone (2, 3). The results indicate that normal regulation of prolactin and progesterone receptors may be altered within PDMT cells. The molecular mechanism of action of prolactin and progesterone on mammary gland growth process is still unknown. Nevertheless, the striking growth responsiveness of PDMT to mammogenic hormones, in particular prolactin and progesterone, may be a function of altered prolactin and/or progesterone receptor regulation within certain populations of mammary epithelial cells during the physiologic state of pregnancy.

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