

Prolactin and Estrogen Binding in Transplantable Hormone-dependent and Autonomous Rat Mammary Carcinoma¹

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SUMMARY

A hormone-dependent subline of the transplantable rat mammary tumor MTW9 contains binding sites for both prolactin and estrogen. Prolactin binding is saturable ($K_d \sim 2 \times 10^{-9}$ M), hormone specific, and destroyed by proteases. By contrast, an autonomous subline derived from the same parent tumor has lost more than 75% of both prolactin- and estrogen-binding sites, although binding affinities for both hormones are unchanged. This reduction in binding sites for both prolactin and estrogen in the autonomous line may result in an incomplete recognition of the tumor cells as a target for the circulating hormones with a subsequent loss of hormone-dependent growth characteristics.

INTRODUCTION

Investigations into the mechanism of hormone dependence in mammary carcinoma have clearly identified estrogen and prolactin as the 2 major hormones responsible for growth regulation. It appears that prolactin is the prime stimulus for breast tumor growth but that estrogen-mediated events are necessary for prolactin to exert its influence. Regressions then occur if circulating prolactin levels are reduced by hypophysectomy or if estrogen levels are reduced by ovariectomy (7).

Autonomous breast tumor growth in certain experimental systems has been associated with the loss of certain key components of the biochemical pathways necessary for hormone action in normal target tissues. The loss of one such component, cytoplasmic estrogen receptor, has received considerable attention and these studies have recently been reviewed (8). Prolactin-binding sites, on the other hand, have only just been demonstrated in mammary tumors (2, 3, 14), and their role in breast tumor growth and regression remains to be elucidated.

In this study we have investigated 2 distinctly different transplantable mammary tumors that were originally derived from the same parent tumor, MTW9 (6). One of the tumors, MA, grows equally well in intact, ovariectomized, or hypophysectomized rats and is thus clearly autonomous. The other tumor, MD, also grows in intact rats but

promptly regresses following ovariectomy and thus is hormone dependent. We now show that the autonomous line is markedly deficient in binding sites for both estrogen and prolactin. The resulting failure to be recognized by circulating hormones as a target may explain the loss of hormone dependence in the MA line.

MATERIALS AND METHODS

Mammary tumors were serially transplanted in 180- to 200-g virgin female Wistar-Furth rats (ARS/Sprague-Dawley, Madison, Wis.) by implanting a 2- to 4-cu mm fragment s.c. into the inguinal region. As soon as tumors were palpable (20 to 40 days), growth was measured with calipers at least once a week with the rats under light ether anesthesia. Tumors were routinely used when they reached a size of 2 to 4 sq cm (length x width). Hypophysectomies were kindly performed by Dr. Masataka Shiino. Hypophysectomized rats were maintained on 1% glucose and did not gain weight over the course of the experiments.

Two transplantable tumor lines were used in this study. One of the lines, designated MA, was originally derived from the MTW9 tumor. This line was obtained from Dr. Untae Kim and was classified as an autonomous adenocarcinoma (U. Kim, personal communication). The other line, designated MD, was derived in our laboratory from a prolactin-dependent MTW9 tumor originally obtained from Dr. Vincent Hollander.

Preparation of Tumor Cytosol. Following cervical dislocation, tumors were excised, freed of adhering debris, and frozen immediately in liquid nitrogen. Tissue was stored at -70° until use. To prepare the cytosol fraction, tissue was equilibrated at 4° and homogenized with a Dull homogenizer in TE³ buffer, approximately 2 ml buffer per g of tissue. The homogenate was then centrifuged at $159,000 \times g$ for 45 min to obtain the supernatant cytosol.

Cytosol EB Assay. The concentration of EB in tumor preparations was determined by incubating cytosol with increasing quantities of [³H]estradiol (0.015 to 0.2 pmole) and using the dextran-coated charcoal technique as previously described (9). Sucrose density gradient analysis of estradiol binding in tumor cytosol was performed as described (9).

Nuclear EB. For the localization of EB in tumor nuclei,

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³ The abbreviations used are: TE, 10 mM Tris-HCl-1.5 mM EDTA, pH 7.4; EB, estrogen-binding sites.

animals were ovariectomized 72 hr prior to use. The tumor was excised 1 hr after the rat was given an i.p. injection of [^3H]estradiol (0.1 μCi dissolved in 50 μl ethanol and brought to 0.5 ml with 0.15 M NaCl). Tumor nuclei were prepared from freshly excised tumor by homogenization in TE buffer containing 0.5 mM dithiothreitol and 10^{-6} M unlabeled estradiol. The homogenate was centrifuged at $100,000 \times g$ for 50 min, and the crude nuclear pellet was washed once with 4 ml of the same buffer. The washed pellet was then suspended in 0.6 ml 0.4 M KCl in TE buffer, homogenized in a Duall homogenizer, and extracted at 4° for 75 min. The nuclear extract was centrifuged at $100,000 \times g$ for 15 min, and the supernatant fraction was applied to a 5 to 20% continuous sucrose gradient containing 0.4 M KCl and 0.5 mM dithiothreitol in TE buffer. The gradient was centrifuged in a Beckman SW56 rotor for 16 hr and 45 min at $290,000 \times g$ and analyzed for radioactivity (9).

Iodinated Prolactin. Ovine prolactin was labeled with ^{125}I using a soluble lactoperoxidase method (13). The iodinated hormone was separated from free ^{125}I by G-25F gel filtration and purified by DEAE-cellulose chromatography as described elsewhere (2). Specific activity of the labeled hormone ranged from 55 to 80 $\mu\text{Ci}/\mu\text{g}$ protein.

Prolactin Binding to Tumors. Freshly excised tumors were freed of debris and then sliced to uniform thickness with a Stadie Riggs tissue slicer (0.5 mm) and placed immediately in ice-cold Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing 0.1% bovine serum albumin. Sections of approximately 5 to 10 mg, wet weight, were cut from selected slices and 5 such sections were incubated for 3 hr at 22° in 1 ml of Medium 199 containing 0.1% bovine serum albumin, 5 mM CaCl_2 , and 5 mM *N*-2-hydroxyethylpiperazine-*N*-2'-ethanesulfonic acid buffer and various concentrations of ^{125}I -prolactin as described previously (2). Under these conditions, binding was directly proportional to slice weight for both tumors. Slices were then washed and assayed for radioactivity in a Nuclear-Chicago γ counter, and their wet weights were determined.

Miscellaneous. Protein estimations were according to the method of Lowry *et al.* (5). DNA content was assayed by the procedure of Burton (1) after extraction by the technique of Schneider (12). Insulin was obtained from Sigma Chemical Co., St. Louis, Mo.; epinephrine was from Nutritional Biochemicals Corp., Cleveland, Ohio; all other hormones were kindly supplied by National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

RESULTS

Tumor Growth. Chart 1 shows the growth pattern of the MA tumor in intact, ovariectomized, or hypophysectomized rats. The tumor grows equally well under all these conditions and therefore can be classified as autonomous. Tumor tissue from a hypophysectomized rat was transplanted into ovariectomized animals, and the resulting tumors were used for the experiments performed. Chart 2 is a representative growth pattern of the MD mammary tumor. In contrast to the MA, this tumor grows in intact rats but regresses

promptly following ovariectomy. This tumor is clearly distinct from the MTW9 parent line, which requires very high levels of prolactin for growth and does not regress after ovariectomy (6). We have used the MD line as our model of hormone dependence. The line was serially transplanted from intact animals only, and response to ovariectomy was determined in control rats that received a portion of the same tumor at transplantation.

Histological examination of the MA and MD tumors indicated that they were adenocarcinomas.

Prolactin Binding to Tumors. Competition for ^{125}I -prolactin binding by unlabeled prolactin in both the MA and MD tumors is shown in Chart 3. With excess of unlabeled hormone, total ^{125}I -prolactin binding is diminished by 85 and 70% for the MD and MA tumors, respectively. Although similar competition for prolactin binding is observed for both tumors, the MA line bound considerably less ^{125}I -prolactin in the absence of competitor, suggesting fewer binding sites for the hormone. Tissue from the MD tumor was used to determine whether the observed binding

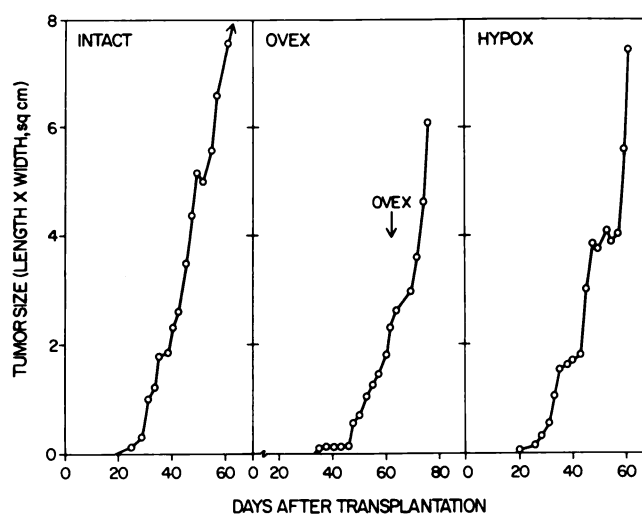


Chart 1. Growth pattern of the autonomous (MA) mammary tumor in intact, ovariectomized (*ovex*), and hypophysectomized (*hypox*) rats.

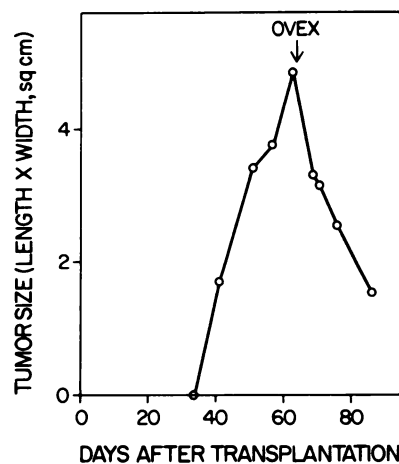


Chart 2. Growth pattern of the hormone-dependent (MD) mammary tumor. An ovariectomy (*ovex*) was performed at the time indicated by the arrow.

had some of the characteristics of a polypeptide hormone receptor. Table 1 shows that the binding sites are specific in their recognition of prolactin. Both unlabeled ovine and rat

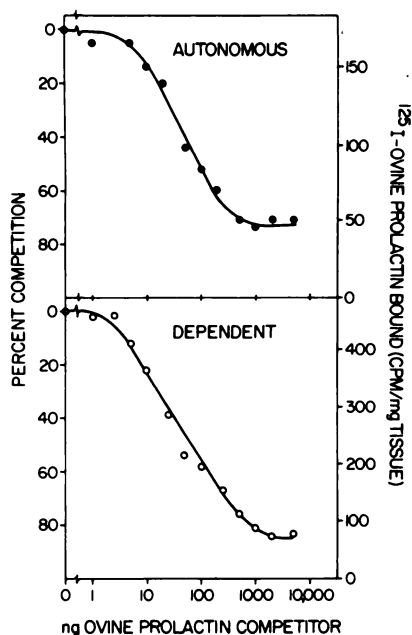


Chart 3. Competition for ¹²⁵I-prolactin binding in the autonomous (MA) and hormone-dependent (MD) mammary tumors. Tumor slices were incubated with ¹²⁵I-prolactin (200,000 cpm) and increasing amounts of unlabeled ovine prolactin for 3 hr at 22°. After the tissue was washed, bound radioactivity and wet weight were determined. Each point is the mean of 5 slices.

prolactin, when used at concentrations with similar biological activity, show the same degree of competition for ¹²⁵I-prolactin binding. The other hormones fail to compete significantly at either concentration. Specific prolactin binding is completely abolished by pretreatment of the tumor tissue with trypsin but not by neuraminidase, hyaluronidase, collagenase, RNase, DNase, or phospholipases A, C, and D, indicating that the prolactin-binding site is protein in nature.

Prolactin binding in the tumors was also examined by Scatchard analysis (11) (Chart 4). In both tumors, prolactin binds to a single class of binding sites with a K_d of ~2 × 10⁻⁹ M. However, the number of binding sites in the MA tumor is only 1.0 fmole/mg tissue, as compared to 4.2 fmoles/mg in the case of the MD tumor. Table 2 indicates the range of values obtained from Scatchard analysis of binding data from a number of tumors. Clearly, the MA tumor contains much fewer prolactin-binding sites than the MD. Determination of DNA content for each sample indicates that prolactin-binding site content is 1.01 ± 0.58 and 0.15 ± 0.19 fmoles/μg DNA for the MD and MA tumors, respectively. Thus, the number of binding sites in the MD tumor is similar to that previously reported for normal rat mammary tissue (2).

EB. Chart 5 illustrates a typical sucrose density gradient profile of cytosols from the MA and MD tumors incubated with [³H]estradiol. For the hormone-dependent tumor, 2 binding peaks are evident. Competition by excess unlabeled estradiol eliminated the 8 S peak entirely and abolished most of the 4 S peak, indicating the specificity of [³H]estradiol binding (not shown). In the autonomous tumor

Table 1

Hormone specificity of ¹²⁵I-ovine prolactin binding to the MD tumor

Slices of tumor tissue were incubated with 200,000 cpm ¹²⁵I-prolactin in the presence of various unlabeled hormones for 3 hr at 22°. After washing, both bound radioactivity and wet weight of tissue were determined. Rat prolactin was adjusted to equivalent biological activity of ovine prolactin.

Hormone competitor	Amount of competitor (ng)	¹²⁵ I-Prolactin bound (cpm/mg tissue)	% competition
None		346 ± 12 ^a	0
Ovine prolactin	50	207 ± 21	40
	2000	77.2 ± 8.2	78
Rat prolactin	137	185 ± 16	47
	5460	72.9 ± 2.8	79
Rat follicle-stimulating hormone	50	294 ± 13	15
	2000	308 ± 7.5	11
Rat growth hormone	50	291 ± 30	16
	2000	269 ± 35	22
Insulin	50	303 ± 20	12
	2000	307 ± 34	11
L-Epinephrine	50	300 ± 27	13
	2000	303 ± 25	12
Ovine luteinizing hormone	50	297 ± 17	14
	2000	287 ± 42	17

^a Mean ± S.E. for 5 slices.

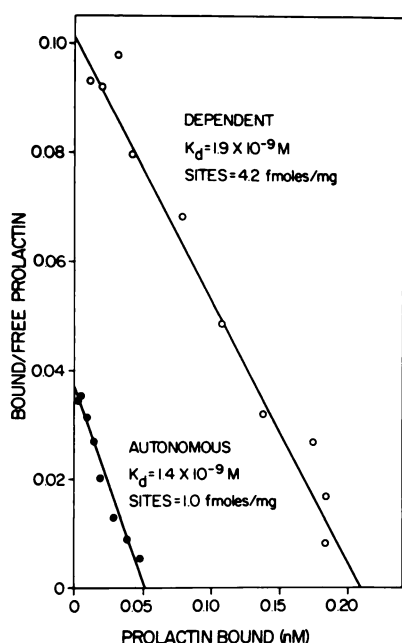


Chart 4. Scatchard analysis of ¹²⁵I-prolactin binding to autonomous (MA) and dependent (MD) mammary tumors. Conditions are the same as in Chart 3.

Table 2

Prolactin-binding site and EB content of MA and MD tumors

Content of prolactin-binding sites and EB was determined by Scatchard analysis of binding data for each tumor as illustrated in Charts 4 and 6.

Experiment	EB (fmoles/mg protein)		Prolactin-binding sites (fmoles/mg tissue)	
	MD	MA	MD	MA
1	51 ^a	7.9	3.7	1.0 ^a
2	71 ^a	9.3	6.5	0.5 ^a
3	87 ^a	6.4	3.1	0.8
4	31	3.2 ^a	4.1 ^a	1.3
5	25 ^a	4.2 ^a	4.1 ^a	0.4
6		4.2		0.4
Mean	53	5.8	4.3	0.73

^a Intact tumor-bearing rat; all others ovariectomized at least 3 days prior to sacrifice.

cytosol, similar results were obtained except that the amounts of [³H]estradiol bound in both the 8 S and 4 S regions of the gradient were considerably lower. Scatchard analysis of binding data from a dextran-coated charcoal assay of cytosols from both tumors is represented in Chart 6. It can be seen that the affinity of the binding sites for estradiol in both tumor cytosols is the same, $K_d \sim 0.5 \times 10^{-10}$ M. In this case EB content is reduced from 51 fmoles/mg protein in the MD tumor to 7.8 fmoles/mg in the MA line. Table 2 shows the results obtained from a number of tumors of each type. The mean reduction in the cytosol-binding sites in the MA as compared to the MD tumor is ~8-fold.

The ability of EB in both tumors to translocate estradiol into the nucleus appears unimpaired in both, as illustrated in

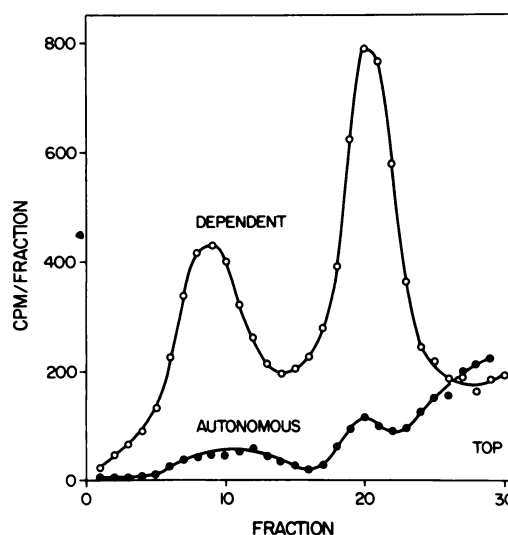


Chart 5. Sucrose density gradient profile of [³H]estradiol binding to cytosols from the autonomous (MA) and dependent (MD) mammary tumors. Under these conditions [¹⁴C]bovine serum albumin (4.6 S) migrates to Fractions 18 to 19.

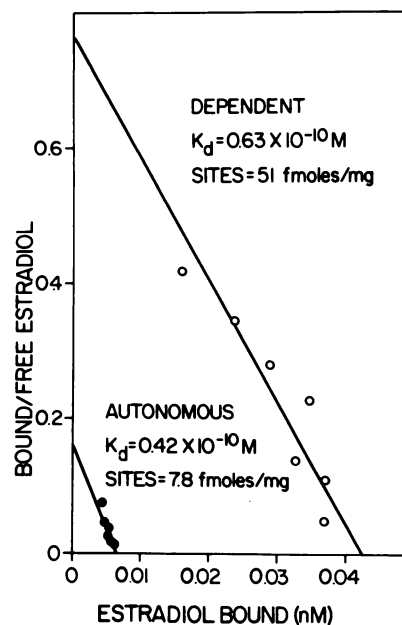


Chart 6. Scatchard analysis of [³H]estradiol binding to autonomous (MA) and dependent (MD) mammary tumor cytosols.

Chart 7. Bound estradiol could be extracted from the nuclei and migrates at ~4 S. The amount of bound estradiol per mg DNA in the MA tumor is lower, reflecting the much reduced cytosol receptor content.

DISCUSSION

Normal mammary cells contain cytoplasmic and/or membrane binding sites for each of the hormones known to influence the growth and function of the mammary gland. These binding sites are responsible for the initial interaction

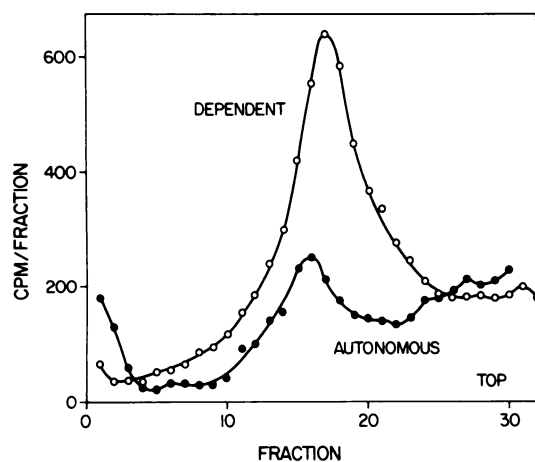


Chart 7. Sucrose density gradient profile of [³H]estradiol binding to nuclear extracts prepared from autonomous (MA) and dependent (MD) mammary tumors 1 hr following injection of labeled estrogen into tumor-bearing rats. [¹⁴C]Bovine serum albumin (4.6 S) was included as an internal standard. The peak was in Fractions 16 to 17.

between the hormone and the cell and trigger the biochemical chain of events characteristic for the particular hormone. When malignant transformation occurs, the cell may retain all or only part of the normal population of binding sites. If the cell retains the binding sites, its growth and function are potentially capable of being regulated by the normal hormonal environment as in a normal cell; however, if the sites are lost from the cell as a consequence of malignant transformation, the cell is no longer recognized as a target cell by the circulating hormones and endocrine control over the inherent unrestrained growth of the cancer cell is absent. This may explain why the MA tumor fails to respond to ovariectomy or hypophysectomy.

We previously reported that the R3230AC rat mammary tumor, which does not regress after ovariectomy, has a normal complement of prolactin-binding sites but is deficient in EB (2, 10). The presence of prolactin-binding sites in the absence of estrogen binding can therefore be associated with autonomous growth. We would anticipate that a normal complement of EB in the absence of prolactin-binding sites would similarly lead to autonomy.

In this report, we have demonstrated hormonal autonomy associated with a marked reduction in binding sites for both prolactin and estrogen. Are they lost by independent mechanisms related to dedifferentiation or selection of certain cell types, or could the loss of 1 binding site lead to the loss of another? There are no direct data on this point, but Vignon and Rochefort (15) have demonstrated a marked reduction in EB levels in hormone-dependent DMBA tumors following ovariectomy. Since prolactin injections restored the tumor EB levels to their previous levels, a possible relationship was suggested. It is unclear, however, whether prolactin specifically stimulated EB synthesis or simply reversed the tumor regression and permit-

ted the EB-containing cells to remain viable. A similar report on the *in vitro* effect of prolactin enhancing estradiol uptake into uterine and mammary explants is subject to the same argument (4). Now that several studies suggest that the loss of endocrine control is closely related to the loss of hormone-binding sites, investigation into the circumstances leading to the loss of these receptors may reveal other mechanisms by which cell function is regulated.

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