Prolactin Binding in Ovariectomy-responsive and Ovariectomy-nonresponsive Rat Mammary Carcinoma

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SUMMARY

Growth of the transplantable mammary tumor, MTW9, in W/Fu rats is greatly enhanced by elevated serum prolactin concentrations. This report compares the prolactin binding to tumor membranes in two mammary tumor strains derived from MTW9. Maximum binding to membranes of both tumors occurred at pH 7.6 after incubation for 30 hr at 4°. The binding was inhibited only by polypeptide hormones that possess lactogenic activity. MTW9-P, an ovariectomy-responsive tumor developed in rats maintained on daily perphenazine injections, had 4-fold-higher prolactin binding than had MTW9-MT, an ovariectomy-nonresponsive tumor developed in rats bearing the mammosomatotropic pituitary tumor, MTW10. Withdrawal of perphenazine from rats bearing MTW9-P caused a fall to normal of plasma prolactin, no tumor regression, and no significant change in prolactin binding. In contrast, resection of MTT resulted in tumor regression, a fall to normal of serum prolactin, and a nearly 3-fold increase in prolactin binding. Scatchard plots of prolactin binding data yield an apparent affinity constant, \( K_a \), of \( 1.2 \times 10^8 \) liters/mole for both tumors.

The 4-fold-higher prolactin binding in the ovariectomy-responsive variant suggests a positive correlation between ovariectomy response and the number of membrane prolactin-binding sites. No correlation between prolactin sensitivity and prolactin binding is apparent.

INTRODUCTION

Growth of the transplantable mammary tumor, MTW9, in W/Fu rats is greatly enhanced by elevated serum prolactin concentrations (4). MTW9 will grow in rats coimplanted with MTW10, a mammosomatotropic pituitary tumor, or in rats maintained on daily perphenazine (4-[3-(2-chlorophenoxy)-1-piperazinemethanol] injections. Both of the above conditions have been shown to raise serum prolactin concentrations to well above normal. The resulting mammary tumors behave differently in response to endocrine manipulation. MTW9-P regresses after ovariectomy; MTW9-MT regresses after MTI resection but not after ovariectomy (4). When perphenazine administration is discontinued, serum prolactin returns to normal; MTW9-P does not regress but is still ovariectomy responsive (4).

Several investigators (1, 15, 19) have reported that the amount of prolactin binding in various tissues, both normal and tumorous, could be directly related to the degree of prolactin dependence of such tissue. In view of the differing response of these 2 tumor sublines to ovariectomy, we investigated the prolactin-binding capacity of these tumors to determine whether a correlation exists between ovariectomy response, prolactin sensitivity, and prolactin binding.

MATERIALS AND METHODS

The source of W/Fu rats, methods of tumor transplantation, surgical procedures, and perphenazine administration have been described previously (4, 10).

Hormones and Chemicals. Ovine prolactin was supplied by the National Institute of Arthritis and Metabolic Diseases, NIH. Rat prolactin was obtained through the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis and Metabolic Diseases, NIH. Human placental lactogen was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Porcine growth hormone and lactoperoxidase were obtained from Calbiochem, Los Angeles, Calif. Porcine insulin and glucagon were generously supplied by Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, Ind. Adrenocorticotropic hormone was a gift of Geigy Pharmaceuticals, Ardsley, N. Y., by courtesy of Dr. J. J. Chart. Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. All other chemicals were reagent grade.

Radioiodination of Ovine Prolactin. Ovine prolactin was iodinated by using a soluble lactoperoxidase procedure slightly modified from that of Gelato et al. (6). Purification was accomplished by chromatography on Sephadex G-75 instead of Sephadex G-50. The procedure consistently yielded a radioiodinated product with a specific activity range of 100 to 150 \( \mu \text{Ci} \), per \( \mu \text{g} \) ovine prolactin.

Preparation of Tumor Membranes. Crude tumor or liver membranes were prepared according to the method of Shiu et al. (18). Tumor membranes were prepared when the tumor had attained an average diameter between 1 and 2 cm between 1 and 2 cm. Mammary tumors that had regressed to approximately one-half their original size, as a
result of either MtT resection of MTW9-MtT-bearing animals or ovariectomy of animals bearing MTW9-P, were used for membrane preparation. Crude membranes prepared in this manner were stored frozen and showed an unchanged binding capacity after 2 months.

**Prolactin Binding Assay.** The assay used to measure specific prolactin binding was carried out in polypropylene microtubes in a total volume of 150 μl. All additions and dilutions were made with 0.025 M Tris-HCl (pH 7.6), which was 10 mM in CaCl₂ and 1% in bovine serum albumin. The membrane suspension was diluted to contain between 100 and 200 μg of protein per 100 μl, as determined by the method of Lowry et al. (8). Approximately 200,000 cpm of ¹²⁵I-labeled prolactin were added, and incubation was carried out for 24 hr at 4°C. Incubations were terminated by the addition of 300 μl of cold buffer and centrifugation for 5 min in a Brinkmann 3200 centrifuge (12,000 × g). After a 2nd washing and centrifugation, the tubes were blotted with thin strips of filter paper and counted in a Beckman 300 gamma counter. Specific binding is defined as the difference in the amount of ¹²⁵I-labeled prolactin bound in the presence and absence of a 2000-fold excess of unlabeled ovine prolactin.

**RESULTS**

Charts 1 and 2 show the time course of binding at 4°C, 24°C, and 37°C for MTW9-MtT and MTW9-P, respectively. Maximum binding to membranes of both tumors occurred at 4°C after approximately 30 hr of incubation. The binding at 37°C, shown in the insets was approximately 30% lower and was completed within 3 hr. Subsequent assays were performed at 4°C for 24 hr, for convenience and because agreement among replicate assays was generally more acceptable under these conditions. Although specific binding is slightly less at 24 than at 30 hr, the striking differences in binding between the 2 types of tumors were not altered. Maximum binding to both tumor membrane preparations occurred at pH 7.6 (Chart 3). Maximum binding to MTW9-PD membranes occurred at the same time, temperature, and pH (data not shown). Binding to both membrane preparations increased linearly with increasing protein concentration up to about 500 μg membrane protein.

Chart 4 illustrates competition between ¹²⁵I-labeled prolactin and unlabeled ovine prolactin for the 2 membrane receptors studied. Binding was sensitive to prolactin concentrations of less than 10 ng/ml and showed about 15% competition at a concentration of 5 ng/ml. Incubation with greater than 2 μg ovine prolactin per ml produced no further inhibition of labeled prolactin binding. In this experiment approximately 70% of the total binding was specific.

Further studies on hormonal inhibition of labeled prolactin binding to mammary tumor membranes are shown in a representative experiment in Table 1. Of the hormones...
tested, only ovine prolactin, rat prolactin, and human placental lactogen (hormones that possess lactogenic activity) competed significantly with the labeled prolactin for binding to tumor membranes. In addition, Table 1 shows that hormones effective at displacement showed similar affinity for both membrane preparations.

Hyperbolic binding curves for prolactin binding to membranes of the 2 mammary tumors (Chart 5) show saturation at about $1.2 \times 10^{-8}$ M prolactin. Increasing the concentration beyond $1.2 \times 10^{-8}$ M resulted in no apparent significant increase in binding. However, the data were not completely reliable, because nonspecific binding was very high and replicate determinations did not agree well at such high concentrations.

Chart 6 casts these binding data into the form of a Scatchard plot (17). The slopes are nearly identical which is consistent with an apparent $K_a$ of approximately $1.2 \times 10^6$ liters/mole. However, the maximum binding capacity, $n$, is about 3.5-fold greater for MTW9-P than it is for MTW9-MtT.

Table 2 compares prolactin binding to tumor membranes from MTW9-MtT with those prepared from MTW9-P and from both tumors under different experimental conditions. Mammary tumors from animals maintained on perphenazine had a 4-fold-greater binding capacity than did tumors from MtT-supported animals. When perphenazine administration was stopped, the binding capacity of MTW9-P membranes was not significantly altered. The same result held true when MTW9-P- or MTW9-PD-bearing animals were subjected to ovariectomy. However, sham-operated MTW9-PD-bearing rats had tumors with significantly higher prolactin-binding capacities.

Resection of MtT from MTW9-MtT-bearing animals resulted in about a 2-fold increase in prolactin binding, whereas tumors from sham-resected animals had values equivalent to those of MTW9-MtT. Neither host ovariectomy

<table>
<thead>
<tr>
<th>Competitor</th>
<th>MTW9-MtT</th>
<th>MTW9-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine prolactin</td>
<td>86</td>
<td>84</td>
</tr>
<tr>
<td>Rat prolactin</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>Human placental lactogen</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>Porcine growth hormone</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Porcine glucagon</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Porcine insulin</td>
<td>4.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1

Inhibition of $^{125}$I-labeled prolactin binding to mammary tumor membranes by unlabelled competitor

Tumor membranes were incubated with approximately 150,000 cpm of $^{125}$I-labeled prolactin and increasing concentrations of unlabeled ovine prolactin for 24 hr at 4°. Ordinate, percentage of competition by the indicated amount of unlabeled ovine prolactin. Each point is the mean of triplicate determinations.

O, MTW9-MtT; ∆, MTW9-P.

Chart 4. Competition by unlabelled ovine prolactin for prolactin-binding sites in MTW9-MtT and MTW9-P membranes. Tumor membranes (125 μg) were incubated with approximately 250,000 cpm of $^{125}$I-labeled prolactin and increasing concentrations of unlabeled ovine prolactin for 24 hr at 4°. Ordinate, percentage of competition by the indicated amount of unlabeled ovine prolactin. Each point is the mean of triplicate determinations.

Chart 5. Binding of $^{125}$I-labeled prolactin to MTW9-MtT and MTW9-P membranes as a function of increasing concentrations of $^{125}$I-labeled prolactin. MTW9-MtT (O) and MTW9-P (∆) membranes (100 μg protein) were incubated with increasing concentrations of $^{125}$I-labeled prolactin (abscissa) in the presence or absence of a 2000-fold excess of unlabeled ovine prolactin for 24 hr at 4°. Ordinate, amount of $^{125}$I-labeled prolactin specifically bound. Each point is the mean of triplicate determinations.

Chart 6. Scatchard plot of data presented in Chart 5. Ordinate, ratio of bound to free (B/F) hormones; abscissa, amount of ovine prolactin bound to MTW9-MtT (O) or MTW9-P (Δ) membranes.

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DISCUSSION

This study involves an established mammary tumor that, when it is transplanted into hosts with different endocrine states, grows out as tumor sublines with predictable bioreponse to ovariectomy and number of prolactin-binding sites. MTW9 was selected for study, because the basic line has been stable for many years with a requirement for high serum prolactin and because the line does not regress on ovariectomy (4). These transplantable tumor sublines have been found to have a positive correlation between specific prolactin binding sites and the degree of prolactin receptor content (2), this study showed the lowest binding in tumors exposed to the highest serum prolactin concentration. If serum prolactin is approximately $5 \times 10^{-8}$ M (1000 ng/ml) and receptor has a $K_d$ of $10^9$ liters/mole, one would expect that more than 90% of the total receptor would bind ligand. Under these conditions MTW9-MtT would show few available receptor sites for labeled prolactin. This explanation for the apparent low receptor content of MTW9-MtT is consistent with the nearly 3-fold increase in receptor after resection of MtT. However, the lack of change in receptor content observed when rats bearing MTW9-P were removed from perphenazine treatment demonstrates that the difference in receptor content between MTW9-MtT and MTW9-P did not result from receptor sites filled with endogenous prolactin. When perphenazine treatment was stopped, serum prolactin concentration became normal within 24 hr (4). The serum concentration of prolactin in rats under the influence of perphenazine administration is approximately $2 \times 10^{-8}$ M; one would still expect that more than 90% of binding sites would be occupied by endogenous hormone. However, MTW9-P has 4 times more available prolactin-binding sites than MTW9-MtT, and a decrease of serum prolactin to the normal range does not change the number of available sites. Presumably, endogenous prolactin is lost from receptors during membrane preparation in the same manner as is endogenous insulin (16). The difference in prolactin receptor content is reproducible in all transplant generations and is probably a result of some MtT-mediated change in endocrine host status rather than a change in serum prolactin. MtT growth produces high serum prolactin, growth hormone (5), insulin (12) and, presumably, progesterone. This would probably also result in low serum gonadotropin and estrogen. The metabolism of liver, thyroid, mammary gland, and adipose tissue was markedly affected by this increased hormone secretion by the pituitary tumor (9-11).

The number of mammary tumor prolactin-binding sites might be expected to be a function of serum estradiol concentration. Estradiol increased prolactin receptor content in liver (14). However, physiological estradiol concentration did not affect the prolactin receptor content of MTW9. Ovariectomy of rats bearing either MTW9-P or MTW9-MtT had no effect on prolactin binding to tumor membrane. Sham ovariectomy has routinely been done in our laboratory as a control for ovariectomy effect. Sham

after resection of MtT (4). The difference in binding between MTW9-MtT from which MtT has been resected and MTW9-PD was less than 2-fold but still significant (Table 2). MTW9-P did not grow faster when serum prolactin was normal; perhaps tumor growth depends on high serum prolactin, but regression depends on factors other than the number of prolactin-binding sites alone. At present, we believe that in the absence of prolactin-binding sites prolactin cannot act, but there is no correlation between the number of prolactin-binding sites and the degree of prolactin response.

The effect of serum prolactin on tumor hormone receptor measurement is of interest from the standpoint of receptor induction and occupancy by endogenous hormones. Roth (16) has shown that chronic high serum concentrations of insulin cause decreased receptor concentration. Although prolactin is known to induce prolactin receptor in liver tissue (2), this study showed the lowest binding in tumors exposed to the highest serum prolactin concentration. If serum prolactin is approximately $5 \times 10^{-8}$ M (1000 ng/ml) and receptor has a $K_d$ of $10^9$ liters/mole, one would expect that more than 90% of the total receptor would bind ligand. Under these conditions MTW9-MtT would show few available receptor sites for labeled prolactin. This explanation for the apparent low receptor content of MTW9-MtT is consistent with the nearly 3-fold increase in receptor after resection of MtT. However, the lack of change in receptor content observed when rats bearing MTW9-P were removed from perphenazine treatment demonstrates that the difference in receptor content between MTW9-MtT and MTW9-P did not result from receptor sites filled with endogenous prolactin. When perphenazine treatment was stopped, serum prolactin concentration became normal within 24 hr (4). The serum concentration of prolactin in rats under the influence of perphenazine administration is approximately $2 \times 10^{-8}$ M; one would still expect that more than 90% of binding sites would be occupied by endogenous hormone. However, MTW9-P has 4 times more available prolactin-binding sites than MTW9-MtT, and a decrease of serum prolactin to the normal range does not change the number of available sites. Presumably, endogenous prolactin is lost from receptors during membrane preparation in the same manner as is endogenous insulin (16). The difference in prolactin receptor content is reproducible in all transplant generations and is probably a result of some MtT-mediated change in endocrine host status rather than a change in serum prolactin. MtT growth produces high serum prolactin, growth hormone (5), insulin (12) and, presumably, progesterone. This would probably also result in low serum gonadotropin and estrogen. The metabolism of liver, thyroid, mammary gland, and adipose tissue was markedly affected by this increased hormone secretion by the pituitary tumor (9-11).

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Table 2

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Specific binding (fmol/100 mg protein)</th>
<th>No. (%)</th>
<th>$p^a$</th>
<th>$p^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTW9-MtT</td>
<td>2.9 ± 0.3*</td>
<td>8</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>MTW9-MtT resect$^d$</td>
<td>7.5 ± 0.3</td>
<td>4</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MTW9-MtT sham resect</td>
<td>3.7 ± 0.5</td>
<td>3</td>
<td>NS$^d$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MTW9-MtT OVX</td>
<td>3.7 ± 0.8</td>
<td>4</td>
<td>NS</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MTW9-MtT sham OVX</td>
<td>4.8 ± 1.1</td>
<td>3</td>
<td>NS</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>MTW9-P</td>
<td>12.2 ± 1.3</td>
<td>7</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>MTW9-P OVX</td>
<td>11.3 ± 1.5</td>
<td>3</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>MTW9-P sham OVX</td>
<td>12.2 ± 2.1</td>
<td>3</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>MTW9-PD</td>
<td>12.2 ± 1.7</td>
<td>7</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>MTW9-PD OVX</td>
<td>9.6 ± 0.7</td>
<td>4</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>MTW9-PD sham OVX</td>
<td>17.5 ± 1.2</td>
<td>4</td>
<td>&lt;0.001</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

$^a$ Calculated in comparison to MTW9-MtT.
$^b$ Calculated in comparison to MTW9-P.
$^d$ Mean ± S.E.

$^d$ Resect, MtT resection (membranes prepared 5 to 7 days after surgery); NS, not significant; OVX, ovariectomy (membranes prepared 5 to 7 days after surgery); MTW9-PD, binding to MTW9-P membranes 2 weeks after withdrawal from perphenazine.

nor sham ovariectomy had an effect on prolactin binding to MTW9-MtT membranes.
operation has had no effect on prolactin binding to MTW9-MtT or MTW9-P but, curiously, has produced a very reproducible increase in binding to MTW9-PD. In contrast to rats bearing MTW9-MtT or MTW9-P, rats bearing MTW9-PD pass through all stages of the estrous cycle. Nequin et al. (13) observed that sham ovariectomy produced a rise in serum estradiol. Effects of administration of estradiol and progesterone to mimic the effects of sham ovariectomy are under study. At this time we have no explanation for this significant and reproducible effect.

Cytosol estradiol receptor levels in MTW9-P are higher than those in MTW9-MtT, 4 which indicates a positive correlation between prolactin and estradiol receptor content and response to ovariectomy. We wish to emphasize the reproducible nature of the 2 mammary tumor sublines studied here. The in vivo hormonal response and in vitro prolactin binding of the tumors are consistently predictable within narrow limits. A positive correlation exists between the number of prolactin-binding sites and ovariectomy response. MTW9-P, an ovariectomy-responsive mammary tumor, has high levels of prolactin- and estrogen-binding sites whereas MTW9-MtT, which fails to respond to ovariectomy, has a low number of both binding sites. DeSombre et al. (3) reported similar findings in DMBA-induced mammary tumor, although some tumors had relatively high levels of prolactin receptor and were still unresponsive to ovariectomy.

The development of tumor sublines with predictable hormonal binding characteristics and response to endocrine manipulation allows chemical study of the relationship between these variables. Current studies seek to explain the low prolactin receptor content of MTW9-MtT membranes and the lack of ovariectomy response.

REFERENCES


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