Prolactin Receptors in Human Breast Cancer Cells in Long-Term Tissue Culture

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ABSTRACT

Prolactin receptors have been identified for the first time in a number of human breast cancer cell lines and a normal human breast cell line maintained in long-term tissue culture. Optimal conditions for determining the binding of 125I-labeled human prolactin to these cells were established. Five different tumor cell lines have different content of prolactin receptors ranging from 2,300 to 26,000 sites/cell. All tumor cell lines contained more prolactin receptors than does one normal breast cell line (1700 sites/cell). The prolactin receptors in these human mammary tumor cells not only bind human prolactin but also recognize other lactogenic hormones such as human growth hormone, human placental lactogen, and sheep prolactin, but not animal growth hormone, which are not lactogenic. The affinity (Kd) of binding of human prolactin to these cells is 4 x 10^9 M^-1 (Kd = 2.5 x 10^-10 M). The hormone specificity and affinity for hormone of these human mammary tumor cells are very similar to that found for the rabbit mammary gland. These human mammary tumor cell lines in long-term culture should prove very useful to study the biology of prolactin receptors in living human cells and the role of prolactin in the tumorigenesis of the human breast.

INTRODUCTION

Prolactin-dependent mammary tumors are well known in rodents (29). Membrane receptors for prolactin have been demonstrated in chemically induced mammary tumors in rats (5, 7, 11, 25). However, the exact relationship between prolactin receptor level and dependency on prolactin in the growth of these rat tumors is far from established. Although the involvement of prolactin and other endocrine factors in the development of breast cancer in humans has been appreciated (9, 17, 21), the mechanisms underlying the hormone-dependent nature of human breast tumors remain elusive. Nevertheless, evidence from clinical and experimental studies suggests that some polypeptide hormones (e.g., prolactin, growth hormone, and insulin) and many steroid hormones (e.g., estrogen, androgen, progesterone, and glucocorticoids) may play essential roles in the tumorigenesis of the human breast. While most efforts in the past have been devoted to the studies of the effects of steroid hormones, attempts have been made to define a role for some protein hormones, notably prolactin, in the etiology of human breast cancer. For example, in vitro studies with breast tumor biopsies maintained in culture have demonstrated that prolactin influences the pentose phosphate pathway assessed by histochemical techniques (18). Other investigators also reported in vitro effects on DNA synthesis and α-lactalbumin production of prolactin and insulin on breast tumor biopsy specimens (6, 12, 28). Because biopsy specimens are not suitable for long-term studies of hormonal effects on human breast tumors, some investigators recently have used human tumor cells maintained in long-term tissue cultures. Thus, Lippman et al. (14), by using a line of human tumor cells (MCF-7), were able to demonstrate that the growth of these cells is affected by insulin, estrogen, androgens, glucocorticoids, and progesterone. Likewise, Shafie and Brooks (20) demonstrated that prolactin stimulates estrogen receptors in the same cell line.

Since binding of hormone to its specific receptors is essential in mediating the action of the hormone, it is important to study the biology of the receptors for various types of hormones in human mammary tumors. Knowledge in this area may help to better define the roles of hormones in human breast cancers and the mechanisms of actions of some of these hormones. Again, efforts in the past have been largely devoted to the study of steroid hormone receptors. Recently, a number of reports on the identification of prolactin receptors in membrane preparations of breast tumor biopsy specimens have appeared (8, 16, 27). Human mammary tumor cells maintained in long-term tissue culture can be excellent model systems to investigate the biology of prolactin and other hormone receptors and the mode of action of these hormones. In this report, evidence will be presented demonstrating for the first time that prolactin receptors are present in a number of human mammary tumor lines, as well as one normal cell line, in long-term tissue culture. Some properties of the interaction of prolactin with its receptor will also be described.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. MCF-7 was generously provided by Dr. M. Rich, Michigan Cancer Foundation; BT-474 was a gift of Dr. E. Y. Lasfargues, Institute for Medical Research, Camden, N. J. The following cell lines were obtained through the Cell Culture Bank, EG & G/Mason Research Institute, National Cancer Institute, Rockville, Md.: MDA-MB-231; T-47D; Hs0578T; and HBL-100. All these cell lines have been characterized (see manual from EG & G/Mason Research Institute). A normal rat kidney fibroblast line was obtained from Dr. I. Pastan, National Cancer Institute.

All cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium [with 4 mM glutamine and glucose (4.5 g/liter)] containing 10% fetal calf serum, bovine insulin (10 μg/ml), and penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). Trypsin (0.25%) in HBSS was used for cell

1 This investigation was supported Medical Research Council of Canada Grants MA-6246 and ME-6288 and a grant from the J. A. Richardson Foundation, Winnipeg, Manitoba, Canada.

2 Scholar of the Medical Research Council of Canada.

Received September 18, 1978; accepted August 2, 1979.

The abbreviations used are: HBSS, Hank’s balanced salt solution; hPRL, human prolactin; hPL, human placental lactogen (chorionic somatomammotropin); hGH, human growth hormones; oPRL, ovine prolactin; hCG, human chorionic gonadotropin; hLH, human luteinizing hormone.
passages. Cells were maintained in a humidified atmosphere of 95% air-5% CO₂ at 37°. Culture flasks and dishes were obtained from Corning (Fisher Scientific Co., Winnipeg, Manitoba, Canada); culture medium, trypsin, antibiotics, and fetal calf serum were purchased from Grand Island Biological Co. (Burlington, Ontario, Canada).

Hormone Preparations and Iodination. Purified hPRL (>25 IU/mg) and hPL (6.5 IU/mg) as determined by using ovine prolactin as standard were kindly provided by Drs. H. Friesen and I. Worsley, University of Manitoba. Purified hGH (2.6 IU/mg), ovine prolactin (>26 IU/mg), and oGH (0.56 IU/mg) were obtained from the Endocrine Study Section, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH. Purified hCG (>10,000 IU/mg) and hLH (>10,000 IU/mg) were gifts from Dr. K. W. Cheng, University of Manitoba. Bovine insulin (26 IU/mg) was purchased commercially (Grand Island Biological Co.).

hPRL was iodinated by an enzymatic procedure as described by Shiu and Friesen (23) using Na₁²⁵I. The ₁²⁵I-labeled hPRL was purified by chromatography on Sephadex G-50 superfine. The characterization of iodinated hPRL has been previously detailed (23). The specific radioactivity of the labeled hormone ranged from 55 to 75 µCi/µg. By using excess plasma membranes prepared from pregnant rabbit mammary gland (22, 24), it was shown that approximately 53% of the iodo-hPRL prepared in this manner was bindable to excess receptors (Table 1). The amount of ₁²⁵I-labeled hPRL used in each experiment has been expressed in terms of bindable-labeled hormone.

Determination of Specific Binding of ₁²⁵I-labeled Hormone to Cultured Cells. Four dishes (35 mm) of subconfluent monolayer cells were washed once with HBSS containing 0.1% (w/v) BSA. The washing medium was aspirated, and each dish received 1 ml of the same medium containing a given amount of labeled hormone. The amount of bindable labeled hormone used varied from experiment to experiment, but it was in the range of 4 to 5 x 10⁵ cpm (about 5 ng of hormone). Two of 4 dishes, in addition to containing the labeled hormone, also received 1000-fold excess unlabeled hormone. The dishes were incubated at 20–22° (room temperature) for 6 hr when equilibrium was achieved (Chart 1). At the end of this incubation period, the radioactive medium was aspirated, and the dishes were quickly washed twice with 2-ml portions of ice-cold medium. After the final wash, 0.5 ml of 3% sodium dodecyl sulfate solution was added to each dish to dissolve the cells. The fluid in the dishes was agitated, and the dishes were placed in a tilted position to facilitate complete removal of the dissolved proteins by using a Pasteur pipet. The solutions were transferred to small disposable glass tubes, and radioactivity was determined in a LKB Wallac γ-counter with a counting efficiency of 70% for ₁²⁵I. Specific binding was taken as the total radioactivity bound (mean of duplicates) in the absence of unlabeled hormone minus that bound (mean of duplicates) in the presence of excess unlabeled hormone (10 µg/ml). The latter represents nonspecific binding to cells and culture dish, which ranged from 0.5 to 1.0% of the total labeled hormone added (see Chart 3 legend). For a given experiment, cell number was determined for at least triplicate dishes. To achieve this, cells were detached by trypsin solution (0.25% in HBSS) and counted in a Coulter counter, Model ZBI. The variation of cell number per dish was always less than 10%. Experimental designs varied somewhat for some of the experiments to be described later, and these variations will be described in the legends to the charts.

RESULTS

Optimization of Conditions for the Binding of ₁²⁵I-labeled hPRL by Intact Human Mammary Tumor Cells. Initially, a number of cell lines were screened for prolactin receptor activity. One of these, T-47D, was found to possess the highest prolactin-binding activity (Table 2), and these cells were used to determine the optimal time and temperature for the binding of ₁²⁵I-labeled hPRL. Chart 1 shows that the initial rate of specific binding of ₁²⁵I-labeled hPRL was faster at 37° although the time required to achieve maximum binding at this temperature is not very different from that achieved at 20°. At 4°, the rate of association of ₁²⁵I-labeled hPRL was slow, and maximum

Table 1

<table>
<thead>
<tr>
<th>Source of ₁²⁵I-labeled hPRL</th>
<th>% bindable to fresh rabbit mammary receptors</th>
<th>% precipitated by anti-hPRL serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unused</td>
<td>53</td>
<td>89</td>
</tr>
<tr>
<td>Incubation medium at 20°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without cells</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>With cells</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Incubation medium at 37°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without cells</td>
<td>49</td>
<td>84</td>
</tr>
<tr>
<td>With cells</td>
<td>48</td>
<td>82</td>
</tr>
</tbody>
</table>

Chart 1. Effect of time and temperature on the binding of ₁²⁵I-labeled hPRL to intact T-47D monolayer cells. Procedures for determining specific binding of ₁²⁵I-labeled hPRL has been described in "Materials and Methods." However, the incubation media used in this experiment were buffered by additional N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at 50 mm because HBSS does not possess enough buffering capacity when placed at 37° inside an incubator that contains an atmosphere of 5% CO₂-95% air. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at this concentration has no effect on the binding of ₁²⁵I-labeled hPRL. Each dish contained 9.7 x 10⁵ cells and 4 x 10⁶ cpm of bindable ₁²⁵I-labeled hPRL. Points, specific binding of ₁²⁵I-labeled hPRL, that is, the difference between the mean value of duplicates for total binding and the mean value of duplicates for nonspecific binding.

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binding had not occurred after even 12 hr of incubation. In all subsequent experiments, cells were incubated for 6 hr at 20° because the nonspecific binding at 20° (0.5 to 1%) was always lower than that obtained at 37° (1 to 2.5%). Specific binding of 125I-labeled hPRL achieved with HBSS (containing glucose and 0.1% BSA) was similar to that obtained by using Dulbecco’s modified Eagle’s medium (also with 0.1% BSA). Binding of 125I-labeled hPRL was partially suppressed if fetal calf serum was used in the incubation medium. Furthermore, there was no apparent change in the morphology of the cells as judged by phase-contrast microscopy after 6 hr of incubation at any one of the above temperatures; the cells still excluded trypan blue and continued to grow if replaced with growth medium. Moreover, the free 125I-labeled hPRL that was not bound by the cells after 6 hr of incubation at 20 or 37° was not significantly altered as compared with the 125I-labeled hPRL which had not been exposed to cells in its ability to bind excess rabbit mammary receptors as well as its ability to bind to excess anti-hPRL antibodies (Table 1).

Hormone Specificity of the Prolactin Receptor. To test the hormone specificity of the prolactin receptor, competition curves were obtained by incubating T-47D monolayer cells with a fixed amount of 125I-labeled hPRL and in the presence of increasing concentrations of various hormones. Chart 2 shows that all human lactogens (hPRL, hGH, and hPL) competed with 125I-labeled hPRL for the receptor sites; hPL was about 1% as potent as were hPRL and hGH. Ovine prolactin was almost as effective as was hPRL, consistent with its biological activity that was determined in organ culture experiments using human breast tumor biopsy specimens (12). oGH, which is not a lactogen, was ineffective as a competitor. The displacement of the binding of 125I-labeled hPRL by very high concentration of oGH might have been due to contamination of the hormone preparation with small amounts of oPRL. hCG and hLH were without effect. It is also evident from Chart 2 that the total 125I-labeled hPRL bound 75% of this was displaceable by 1000-fold excess of unlabeled hormone, and therefore, 25% of the total 125I-hPRL bound was nonspecific.

Equilibrium Analysis of Binding of 125I-labeled hPRL to Human Tumor and Normal Mammary Monolayer Cells. For all the cell lines tested, specific binding of 125I-labeled hPRL was a direct function of the concentration of labeled hormone in the medium. For clarity, only the binding of 125I-labeled hPRL to T-47D and MCF-7 as a function of hormone concentration was illustrated in Chart 3A. The proportions of hormone bound gradually decreased as hormone concentrations increased until a plateau was reached (for MCF-7). Due to the higher binding capacity of T-47D cells, a plateau was not observed when 350 fmol of 125I-labeled hPRL was used. It was subsequently found that a binding plateau was observed for T-47D at hormone concentrations greater than 600 fmol/ml (data not shown).

The same binding data were analyzed by the methods of Lineweaver and Burk (13) and Scatchard (19). Again, only the plots for MCF-7 and T-47D are presented in Chart 3B. The intercept on the ordinate gave the reciprocal of maximum binding capacity, and the intercept on the abscissa yielded the reciprocal of Kd. Values presented in Table 2 were all derived from these plots. No heterogeneity of prolactin-binding sites was evident from both the Lineweaver-Burk and Scatchard plots (Chart 3B, inset) for all the cell lines tested.

Summary of Prolactin Receptor Contents in Several Human Mammary Tumor and Normal Cells. The optimal conditions illustrated in Chart 1 were used to quantitate prolactin receptors by Lineweaver-Burk and Scatchard Analyses (Chart 3B) in 5 human mammary tumor cell lines and one normal cell line. Table 2 shows that a tumor cell line designated at T-47D had the highest content of prolactin receptors (25,800 sites/cell). The widely used MCF-7 had about one-third (8310 sites/cell) of that of T-47D. One cell line derived from normal human milk, HBL-100, had the lowest receptor content (1700 sites/cell). The Kd (2.5 x 10^-10 M) is identical for all human cell lines tested. One line of rat kidney fibroblasts exhibited no

**Table 2**

<table>
<thead>
<tr>
<th>Prolactin receptors</th>
<th>Cell line</th>
<th>Kd (M)</th>
<th>No. of receptor sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-47D</td>
<td>2.5 x 10^-10</td>
<td>25,800</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>2.5 x 10^-10</td>
<td>8,310</td>
<td></td>
</tr>
<tr>
<td>BT-474</td>
<td>2.5 x 10^-10</td>
<td>5,480</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>2.5 x 10^-10</td>
<td>3,760</td>
<td></td>
</tr>
<tr>
<td>Hs0578T</td>
<td>2.5 x 10^-10</td>
<td>2,260</td>
<td></td>
</tr>
<tr>
<td>Normal cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBL-100</td>
<td>2.5 x 10^-10</td>
<td>1,700</td>
<td></td>
</tr>
</tbody>
</table>
Chart 3. A, specific binding of ¹²⁵I-labeled hPRL to T-47D and MCF-7 cells as a function of the concentration of bindable labeled hormone. Dishes of cells were incubated for 6 hr at 20° in the presence of increasing concentration of ¹²⁵I-labeled hPRL. For each hormone concentration, duplicate dishes also contained 10 µg of unlabeled hormone to account for nonspecific binding. The nonspecific binding for T-47D ranged from 0.16 femtomol at a hormone concentration of 20 femtomol/dish (10 femtomol bindable hormone) to 4 femtomol at a hormone concentration of 660 femtomol/dish (350 femtomol bindable hormone). The corresponding values for nonspecific binding by MCF-7 are 0.19 and 6 femtomol. Cell number per dish for T-47D was 1.2 x 10⁶ and for MCF-7 was 1.1 x 10⁶. B, equilibrium analyses of the binding of ¹²⁵I-labeled hPRL to T-47D and MCF-7 cells. The data from Chart 3A were used. Details for the application of Lineweaver-Burk and Scatchard plots (inset) for the study of prolactin receptor had been described previously (22). Hb, bound hormone; Hf, free bindable hormone.

DISCUSSION

A number of human mammary tumor cell lines and a normal human mammary cell line were shown to possess specific receptor sites for prolactin. This represented the first time that prolactin receptors had been detected and characterized in human mammary cells maintained in long-term tissue cultures. Lippman et al. (14), at the National Cancer Institute reported that they were unable to detect any prolactin receptor in a number of human mammary tumor cell lines which include MCF-7. Since these investigators provided no details as to how they carried out their binding studies, it would be difficult to explain their failure to detect prolactin receptors. However, it was most probably due to the human prolactin preparation that was used in their study. Many investigators had difficulty in obtaining good iodohuman prolactin even for radioimmunoassay purposes. To obtain iodohuman prolactin for receptor activity.

Dissociation of ¹²⁵I-labeled hPRL Bound to T-47D Cells. Chart 4 depicts the kinetics of dissociation of ¹²⁵I-labeled hPRL from T-47D cells. The rate of dissociation at 0° was slow, with a t¹/₂ of 14.4 hr. At higher temperatures, the dissociation curves were nonlinear. The initial dissociation rate at 20° had a t¹/₂ of 2.7 hr, followed by a slower rate having a t¹/₂ of 7 hr. This nonlinearity was more pronounced at 37°; the first 70% of the bound hormone was dissociated from the cells with a t¹/₂ of about 0.46 hr, and the remaining 30% of the bound hormone dissociated at a slower rate with a t¹/₂ of 1.7 hr. More than 95% of receptor-bound ¹²⁵I-labeled hPRL was released into the incubation medium by 6 hr. Furthermore, the dissociation of ¹²⁵I-labeled hPRL at 37° in the presence of excess (10 µg/ml) unlabeled hormone did not differ significantly from that found in the absence of unlabeled hormone, probably reflecting an absence of the phenomenon which De Meyts (4) described as negative cooperativity.
The properties of the prolactin receptors in these human cell lines are very similar to that of the rabbit mammary tissue (22, 24). It is surprising that even the hormone specificity of the prolactin receptor in the human cells is identical to that of the rabbit. The same hormone specificity was reported by Stagner et al. (27) and Holdaway and Friesen (8) who used membrane preparations derived from human breast tumor biopsy specimens. The observation that hGH shares the same receptor site for prolactin raises an interesting speculation. Assuming that prolactin plays a role in the etiology of some human breast cancers, then lowering prolactin (e.g. by ergot derivatives such as CB-154) alone may produce no effect. It may be essential to lower or remove growth hormone as well (9, 15, 26). The observation that hPRL, hGH, and oPRL occupy the same receptor site in these human mammary cell lines suggests that these hormones may produce the same biological effect. Studies using biopsy specimens maintained in organ culture support this notion (12, 18, 28).

The observation that the dissociation of receptor-bound 125I-labeled hPRL is biphasic suggests that this is not a simple first-order reaction. One possible explanation is that there may be 2 classes of prolactin receptor sites, each having a different rate of dissociation. The failure to observe 2 classes of receptors by equilibrium analyses does not support this notion. However, it is conceptually possible that the 2 classes of prolactin receptors may possess identical hormone-binding characteristics but may differ in their hormone dissociation characteristics. This possibility cannot be ruled out from the present study. Another possibility is that some energy-dependent processes may be involved in the dissociation of receptor-bound prolactin. It has been reported recently that the binding of human chorionic gonadotropin to Leydig cells of the testis (1-3) and that the binding of insulin to fat cells (10) was followed by an energy-dependent release of the degraded hormones. In fact, the dissociation of chorionic gonadotropin is also nonlinear (2), suggesting that prolactin degradation may account for the nonlinearity of the prolactin dissociation curves as observed in these experiments. The observation (Table 1) that the 125I-labeled hPRL recovered from the incubation medium was not significantly altered does not seem to support this notion. However, the failure to observe degraded hormone in the incubation medium might have been due to the fact that only a small portion of 125I-labeled hPRL was degraded in the initial 6 hr of incubation with the cells. This small percentage of degraded hormone, when mixed with a vast excess of intact 125I-labeled hPRL in the incubation medium, might have escaped detection by the procedures used. The possibility of degradation of prolactin by living mammary tumor cells is currently under investigation.

The quantitation of prolactin receptors reported in this work is based on 2 assumptions: (a) that there is no heterogeneity in cell types in any one given cell line that possess prolactin receptors, and (b) that the cell cycle has no effect on the level of prolactin receptors. At present, there is no evidence to suggest that these assumptions are valid. These problems are currently being examined.

All human tumor cells possess more prolactin receptors than does one normal cell line tested. Since only one normal cell line is currently available and tested, it is impossible to conclude whether human mammary tumor cells contain more prolactin receptors than do normal mammary cells. Nevertheless, these human mammary cell lines in long-term culture should prove useful for future studies on the biology of prolactin receptors in these cells and the role of prolactin in the tumorigenesis of the human breast.

ACKNOWLEDGMENTS

The author would like to thank Dr. E. Y. Lastargues (Institute for Medical Research, Montpellier, N. J.), Dr. M. Rich (Michigan Cancer Foundation), and Dr. E. M. Jensen (EG & G/Masson Research Institute, Rockville, Md.) for providing the cell lines used in this study. The technical assistance of B. Berliner and the secretarial help of N. Ryan are very much appreciated. The author extends his appreciation to Dr. I. Worsley for his cooperation and to Drs. H. G. Friesen, R. Carlson, and H. Salih for critically reviewing this paper.

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