Role of Serum in the Prolactin Responsiveness of MCF-7 Human Breast Cancer Cells in Long-Term Tissue Culture

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ABSTRACT

MCF-7 human breast cancer cells, grown in long-term tissue culture, were found to be highly responsive to prolactin in terms of growth even in the presence of serum. Human prolactin, placental lactogen, and growth hormone (50-250 ng/ml) stimulated MCF-7 cells to grow when added to culture medium of cells in the presence of charcoal-stripped serum. Under these same conditions, estradiol-17β at 10⁻⁶ M achieved only a 2-fold increase. After 6 days of culture, both estradiol-17β and prolactin gave a total 5-fold increase in cell number. No prolactin effect was achieved in the presence of 10% fetal bovine serum. Stripping fetal bovine serum with dextran-coated charcoal removes as much as 85% of the endogenous lactogens. Removal of these hormones is essential for demonstration of subsequent prolactin-induced growth response in MCF-7 cells, since bovine prolactin binds effectively to lactogen receptors on the surface of the cells but does not transmit a growth signal. When added simultaneously with human prolactin, bovine prolactin blocks the growth response to the former hormone. These results clearly demonstrate that, under the proper conditions of culture, the human breast cancer cell line MCF-7 is highly responsive to growth stimulation by homologous lactogenic hormones. This then affords us an excellent model for further studies on the possible role of prolactin in growth and maintenance of human breast cancer.

INTRODUCTION

The role of prolactin in the induction and progression of mammary tumors in experimental animals is well established (1-3). However, the function of this lactogenic hormone in the etiology of human breast cancer is still controversial (4). Several laboratories have established the presence of specific prolactin receptors in some human breast cancer biopsies (5-8). Although the presence of prolactin receptors has been established in as much as 70% of all human breast cancer samples (6, 7), no clear clinical response has been found by suppressing the serum prolactin levels in patients with this disease (9). As biopsy samples are not always suitable for long-term tissue culture, a number of cell lines established from breast cancer patients were assayed for the presence of the prolactin receptor. The number of binding sites varies from cell line to cell line (10).

Despite the presence of abundant receptors for lactogenic hormones on these tissue culture cells, a clear and consistent response to prolactin has not been established. While T47D cells have been reported to alter their shape, adhesion, and lipid accumulation in response to hGH (11) and prolactin (11), they are unresponsive in terms of growth (10). Similarly, ovine prolactin (1 µg/ml) is without effect on the CAMA-1 cells (12). However, the newly established line EFM-19 is stimulated to grow in serum-free conditions by human prolactin with other lactogenic hormones giving little or no response (13). Using 734B, a clone of MCF-7, and the BT-20 cell lines, Klevjer-Anderson and Buehring (14) reported a significant decrease in population doubling time with a single pharmacological dose (1 µg/ml) of prolactin in the presence of whole (unstripped) serum. Using the MCF-7 cell line, various other laboratories have reported only negative results for prolactin responses in vitro. Shafie and Brooks (15) reported that prolactin (1-10 µg/ml) increased the number of estrogen receptors in these cells but that no increase in growth rate resulted. Shiu (10) also was unable to demonstrate an effect of either human prolactin or hGH (1 µg/ml) on growth of these cells either in the presence of 1% CSS or under serum-free conditions. Likewise, Jozan et al. (16) reported negative results with ovine prolactin when cells were grown on extracellular matrix under serum-free conditions.

In contrast to the lack of effects in vitro, both T47D and MCF-7 cells respond to lactogen stimulation when grown as solid tumors in nude mice (17-19). This effect was only apparent when the animals either had intact, functioning ovaries or were supplemented with estrogens. Similar results were also reported for primary breast biopsy samples implanted in nude mice (20). These data obtained using nude mice would suggest that prolactin plays a role, either direct or indirect, in the growth of human breast cancer cells. This is further supported by recent reports from several laboratories using primary cultures of mammary epithelial cells isolated from breast biopsy material and reduction mammoplasty samples. Klevjer-Anderson and Buehring (14) reported that pharmacological concentrations of ovine and human prolactin can affect the growth rate of malignant and nonmalignant human epithelial cells. Malarkey et al. (21) found that physiological levels of human prolactin and hGH, but not ovine prolactin, increased the population doubling of primary breast tumor cultures. Similar results were also obtained with benign human breast tissue by Welsch and McManus (22). Hammond et al. (23) report that multiple serial passages of human mammary epithelial cells in serum-free conditions require the presence of bovine pituitary extract. Replacement of the extract with prostaglandin E₁ and ovine prolactin yields a defined medium that allows for three to four serial passages.

Thus, we have reexamined the role of prolactin in the growth of breast cancer cells in long-term culture using the MCF-7 cells as a model. We find that these cells are responsive to physiological concentrations of human lactogens under proper growth conditions, even in serum-containing medium. A possible explanation for the lack of response reported by others is presented based on receptor occupancy.

MATERIALS AND METHODS

Materials. Human prolactin (NIADDK-hPRL-I-6), ovine prolactin (NIADDK-oPRL-17), and human growth hormone (NIADDK-hGH-I-1) were obtained through the Hormone Distribution Program of NIH. Highly purified bovine prolactin and human placental lactogen were the generous gifts of Dr. Keith Moffat, Cornell University, Ithaca, NY (24, 25). E₁ was purchased from Sigma Chemical Laboratories, St.
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Louis, MO. Culture media, antibiotics, trypsin-EDTA solution, trypan blue, and FBS were purchased from GIBCO, Grand Island, NY. BSA (fraction V, pH 7.0, catalogue no. 81-003-2) was purchased from Miles Scientific, Naperville, IL. [125I]hGH was prepared by the lactoperoxidase method as described (26) and had a specific activity of 40–60 µCi/µg. Dextran T-70 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ.

Cell Growth. The human breast carcinoma cell line MCF-7, which was originally isolated from a pleural effusion of a primary breast cancer patient (27), was obtained from the Breast Cancer Task Force (National Cancer Institute, Bethesda, MD) at passage 115. The cell line was routinely subpassaged in Dulbecco's modified Eagle's medium supplemented with insulin (100 U/ml), penicillin (100 U/ml), streptomycin (10 µg/ml), and 10% heat-inactivated FBS. Cells were maintained at 37°C in a 5% CO₂-enriched humidified air atmosphere. For most experiments utilizing hormones, unless otherwise indicated, the cells were grown for 4 days in 10% CSS prior to seeding for growth and binding studies. Hence, all studies were performed on cells cultured in the presence of 10% CSS for at least the previous passage. This minimizes the carry-over of bovine lactogens into that experiment. Cells were seeded in triplicate 6-well tissue culture dishes (35-mm diameter) at an initial density of 45 × 10³ cells/well with 3 ml of media/well. Cells were allowed to attach for 1 day before hormones were added (designated as day 0). Plating efficiency was 85–90%. Cells were harvested at 3 or 6 days, as indicated in Figs. 1–9, legends, by brief trypsinization and counted using a hemacytometer. Cell viability (>95%) was determined by trypan blue exclusion (28). All experiments were performed a minimum of three times.

Preparation of CSS. A modification of the methods of Lippman et al. (29) and Horwitz et al. (30) was used. Briefly, a suspension of 0.01 M Tris-HCl, pH 8.0, containing 0.25% Norit A/0.0025% Dextran T-70 (w/v) was centrifuged at 600 × g to wash the charcoal. For each milliliter of serum to be stripped, 2 ml of charcoal suspension was pelleted. The supernatant was removed by aspiration, the pelleted charcoal was resuspended in serum and incubated at 55°C for 30 min and then centrifuged, and the serum was carefully removed with a pipet so as not to disturb the charcoal pellet. The serum was sterilized using a 0.20-µm pore size filter (Nalgene) and then stored at −20°C until used. A single cycle of serum through dextran-coated charcoal using this method removes up to 85% of the endogenous lactogens in FBS.

Multiple cycles of stripping may be needed to reduce the lactogen concentration further (see "Results").

Determination of Lactogen Content of Serum. The lactogen content of FBS and CSS was determined by a radioreceptor competition method described previously (31) using ovine prolactin as standard. Triplicate aliquots of various dilutions of the sera were incubated overnight at room temperature in the presence of 70,000 dpm [125I]hGH and 200 µg of membrane proteins prepared from livers of lactating mice. Hormone-receptor complexes in the presence of 10 mM MgCl₂ were collected by centrifugation, and radioactivity was determined in a γ-counter. Data given are in ovine prolactin equivalents.

Lactogen Binding to Whole Cells. Triplicate 35-mm dishes of subconfluent monolayers of cells were washed once with PBS containing 0.1% (w/v) BSA. The washing media were removed by aspiration and replaced with PBS/BSA (1 ml) containing 700,000 dpm [125I]hGH in the absence and presence of unlabeled hormone. The dishes were incubated at room temperature for 4 h after which the media were removed by aspiration. Cells were washed twice with 0.5 ml ice-cold PBS/BSA. Each dish then received 0.5 ml of a 3% solution of sodium dodecyl sulfate at room temperature. After gentle agitation to release the cells, the solution was pipeted off and placed in a plastic tube for counting in a γ-counter. Specific binding was calculated as the difference between total binding (no added unlabeled hormone) and nonspecific binding (in the presence of unlabeled hormone).

Statistical Analysis. The significance of the difference between two groups was determined by Student's t test.

RESULTS

Growth of the MCF-7 cells is responsive to the presence of prolactin in the medium under appropriate serum-containing conditions. This is illustrated in Fig. 1. When MCF-7 cells are grown for 3 or 6 days in the presence of 10% FBS containing human prolactin at concentrations ranging from 10 ng/ml to 10 µg/ml, little or no enhancement of cell growth is observed (higher concentration and later time points not shown). This lack of response to prolactin was observed whether or not the cells were preconditioned by growth in 10% CSS for 5 days prior to exposure to the FBS and hormone. The only difference under these two conditions was in the baseline (no hormone) response to the FBS. Preconditioned cells show a lag of 5 or 6 days before they begin to respond to FBS but still show no additional response to prolactin. However, if the cells are continuously exposed to serum which has been stripped of endogenous hormones by pretreatment with dextran-coated charcoal, prolactin-stimulated growth is observed. The prolactin-stimulated growth occurs more rapidly than estrogen-stimulated growth. After 3 days in the presence of 10% CSS, E₂ (10⁻⁸ M) gives a 30% increase in cell number compared with 10% CSS alone (a 2-fold increase over seeding density). Human prolactin (100 ng/ml) on the other hand gives a 2.4-fold increase in cell number over CSS alone and a 4.4-fold increase in cell number over seeding density. After 6 days of culture in the presence of either of the hormones, the same number of cells is present. This represents a 5-fold increase in the number of cells over the number seeded but only 80% more cells than in the absence of added hormones (i.e., 10% CSS alone). Cells exposed to human prolactin for 3 or 6 days in the presence of 10% CSS grow as well as cells continuously grown in the presence of FBS (i.e., not preconditioned with CSS). The prolactin-stimulated growth of these cells is more evident in the presence of 1% CSS compared with 10% CSS. More than a 3-fold increase in cell number is seen after 3 days in the presence of 1% CSS and prolactin compared to 1% CSS alone. This represents a 7.5-fold increase in cell number compared with the seeding density. Under these same conditions, E₂ only gave a 40% increase in cell number (3-fold over seeding density). After 6 days, total cell survival in the presence of 1% CSS, even with hormonal supplements, was significantly decreased (data not shown). Five % CSS gave results similar to those in 10% CSS. The E₂ concentration used (10⁻⁸ M) was maximal for the MCF-7.
7 cells grown in our laboratory. Lowering the E2 concentration only resulted in fewer cells present under all growth conditions tested.

The lack of response of the cells to prolactin in the presence of 10% FBS is probably due to the presence of endogenous lactogens in the serum. Table 1 shows the content of lactogen present in FBS and in CSS after a single cycle of exposure to dextran-coated charcoal. The level of lactogen remaining in the CSS is an important factor in the subsequent response of the cells to prolactin. A single cycle of charcoal stripping removed an average of 75% (range 56–85%) of the endogenous lactogens, reducing the level to below 50 ng/ml. This gives less than 5 ng/ml at a final concentration of 10% CSS. Additional cycles of stripping will reduce the level further but do not significantly alter the experimental results.

The ability of the various lactogens to stimulate growth of the MCF-7 cells in the presence of 10% CSS is shown in Fig. 2. Human prolactin appears to be most effective in the stimulation of growth over a 3-day period. The minimal effective concentration of this hormone in the presence of 10% CSS is 10–50 ng/ml, which is in the physiological range. The maximal response is obtained at 100–250 ng/ml, after which there is a dramatic decrease in the hormone’s effect. The other lactogens which stimulated the cells to grow in CSS (hGH, human placental lactogen, and ovine prolactin) were less effective than human prolactin. While they occasionally gave responses at 50 ng/ml, for consistent responses at least 100–250 ng/ml were required. All lactogens tested showed a decrease in stimulation above 500 ng/ml. The reason for this is unknown. The degree of the decrease varied with each hormone and experiment. Bovine prolactin did not stimulate the cells to grow better than in CSS alone, even at concentrations as high as 500 ng/ml.

The ability of various lactogens to compete for the lactogenic hormone receptor on the surface of the MCF-7 cells is shown in Fig. 3. The lactogens, human prolactin and human placental lactogen, effectively compete with labeled hGH for the receptors, while ovine and bovine prolactins as well as hGH also are potent binders. In fact, it would appear from Fig. 4 that bovine prolactin is a more effective competitor for the lactogenic binding sites on the cells than is human or ovine prolactin.

The bovine prolactin binds to the receptors but does not transmit the signal to grow to the cells. As a result, when bovine lactogens are present in the medium either as the endogenous hormones of FBS (see Fig. 1) or by simultaneous addition to CSS as shown in Fig. 5, no stimulation of the cells is achieved when human prolactin is added at concentrations ranging from 50 to 250 ng/ml. This effect is achieved, at least in part, at bovine prolactin concentrations as low as 50 ng/ml.

**DISCUSSION**

That estrogens are the predominant hormone involved in human breast cancer growth regulation is a well-established
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Fig. 5. Effect of different concentrations of bovine prolactin on MCF-7 cells grown in the absence or presence of different concentrations of human prolactin. MCF-7 cells were grown for 1 day in the presence of 10% CSS before the simultaneous addition of the human and bovine prolactins on day 0. Three days later the cells were harvested by brief trypsinization and viable cells counted by trypan blue exclusion using a hemacytometer. Bovine prolactin was used at 0, 50, 150, or 250 ng/ml. Points, mean of triplicate determinations; bar, mean ± SE.

and accepted fact (32, 33). The possible physiological role of other hormones, particularly prolactin, has not been as well established. Previously published work which attempted to demonstrate that human breast cancer cells in continuous culture can respond to lactogenic hormones has been negative. No consistent response of various cell lines has been shown, even though cells prepared from biopsy samples and from normal breast tissue obtained from reduction mammoplasty appear to respond to prolactin (14, 21–23). A reason for this apparent inconsistency may be found in the results presented in this paper. The MCF-7 human breast cancer cell line was tested for its ability to bind various lactogens and for the ability of these same hormones to stimulate the growth of cells in culture. All of the lactogens tested were able to effectively bind to the cells. In fact, the preparation of bovine prolactin which we used in this study (7) appeared to be more effective in binding to the receptors, in that saturation of the binding sites was achieved at a lower concentration of this hormone than either human or ovine prolactins. Yet, bovine prolactin was unable to transmit a growth signal to the cell. In fact, the simultaneous addition of bovine prolactin and human prolactin to the cultures of MCF-7 cells resulted in significant diminution of the response to the latter hormone even at concentrations of bovine prolactin as low as 50 ng/ml. FBS, which is routinely used in most laboratories to support the growth of the MCF-7 cell, contains significant levels of bovine lactogens or receptor competition activities (34, 35). Most probably, the majority of this is related to bovine placental lactogen, which is responsible for blocking the effect of human prolactin on MCF-7 cells. A similar situation of one hormone blocking the action of a related hormone has also recently been reported using a recombinant form of hGH which is missing 13 amino acids at the amino terminus (Met 14-hGH) (36). This synthetic hormone is unable to elicit a growth response in the rat lymphoma cell Nb2 and blocks the growth response of other active lactogens when added to culture medium simultaneously with them. It also inhibits bovine prolactin-stimulated fat synthesis and α-lactalbumin secretion in explants from bovine mammary glands. Met 14-hGH binds to the prolactin receptors on the cell surface and competes with the prolactins for the binding sites (36). The reason for the significantly different effects of bovine versus ovine prolactins is not as yet clear. One possibility for this disparity may be that, despite similar amino acid sequences, the degree and nature of the glycosylation (37) of the monomeric form of the hormones may vary significantly, thus affecting hormone-receptor interaction and subsequent signal transduction.

CSS has been extensively used in studies on cell growth and responses under the influence of steroid hormones. We demonstrate in this paper that, in addition to removing steroids, dextran-coated charcoal treatment also removes the majority of the lactogenic hormone binding activity. This stripping procedure is essential if a response to exogenous lactogens is being sought. Bovine prolactin blocks the growth response to lactogens from other species even at concentrations as low as 50 ng/ml when added simultaneously and has an apparent advantage in binding to the receptors on the cell surface. Since the cells are initially grown in the presence of CSS for 1 to 5 days before the addition of the hormones, it is essential to remove as much of the endogenous lactogen as possible, even if it means performing multiple cycles of charcoal stripping. The endogenous lactogens in FBS will diminish the subsequent growth response to human prolactin even if CSS is added simultaneously with the hormone. Hence, it is essential that cells be grown in CSS for 1 to 5 days prior to hormone addition for maximum responses. Thus, by reducing the level of endogenous lactogens in the serum, physiological levels of prolactin, such as those used in this study, elicit a growth response in some human breast cancer cell lines (4). Charcoal stripping procedures designed to remove steroids may not remove lactogens. Using serum stripped of steroids, pharmacological levels of prolactin were needed to elicit a response in other breast cancer cell lines (14).

It is important to note that cells preconditioned by 5 days of growth in 10% CSS prior to reexposure to FBS do not initially respond well to the latter in terms of growth (Fig. 1). A 5- or 6-day lag in growth occurs which is not overcome by addition of human or ovine prolactin to the media at levels as high as 10 μg/ml. The reason(s) for this lack of response to the general growth-promoting activity of FBS is not yet clear. Possibly, selection of a different subpopulation of cells occurs since this is not a cloned line, or the cells may become “supersensitive” to the inhibitory effects of the endogenous bovine lactogens in the FBS, or a combination of both events may occur.

The MCF-7 cells in the presence of CSS appear to respond maximally to lactogens of human origin. While ovine prolactin stimulated the cells to grow once the bovine lactogen was removed from the serum, the response was neither as consistent as, nor as great as, that of human prolactin. This observation may explain the negative results reported by some authors using MCF-7 cells and ovine prolactin. The maximal effectiveness of homologous hormonal systems is a well-established endocrinological phenomenon.

One observation which is still unexplained is the decrease in response at high concentrations of human prolactin. This was routinely seen at concentrations of 500 ng/ml and higher and could explain the lack of prolactin-induced growth reported by
several investigators who did use CSS but used human prolactin at 1–10 μg/ml. A similar decrease in response at elevated concentrations of prolactin has been reported by Gertler et al. (38) who studied maintenance of the differentiated state in explant cultures of lactating bovine mammary glands. The lack of response at higher prolactin concentrations may be related to down-regulation of the receptors by the hormone (39).

Prolactin receptors have been found in as many as 70% of human breast cancer samples examined (7). A direct correlation of prolactin receptor status with estrogen/progesterone receptor status or stage of the disease has not been established (40, 41). In addition, we have found that not all human breast cancer cell lines in long-term culture respond to human prolactin in terms of growth (4). In agreement with Shiu (10), we find that the T47D breast cancer cell line, which is reported to have an abundance of prolactin receptors (42), does not respond to human prolactin in terms of growth even in the presence of CSS. Not all of the clones of MCF-7 which we have examined are able to respond to prolactin in terms of growth in the presence of lactogens, even though they have receptors for the hormone. The lack of a growth response by these cells, however, does not necessarily mean a lack of hormone response. Shiu and Paterson (11) have reported a change in cell shape, adhesion, and lipid accumulation by T47D cells in response to human lactogens. These same cells also respond to prolactin with specific induction of a protein which exists in three distinct glycosylated forms (43). Thus, the role of prolactin in the etiology of human breast disease may not be simple, but its role may be a complex multipurpose system which we can only investigate under culture conditions properly staged by removal of endogenous hormones from the culture medium. The use of CSS may point us in the right direction as we develop serum-free conditions in which multiple cell passages with high cell survival can be accomplished. With this tool in hand, it may now be possible to define what, if anything, is the role of prolactin in human breast cancer.

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