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ABSTRACT

A possible autocrine function of prolactin (Prl) in human breast cancer was explored by the addition of a panel of anti-human Prl mAbs to T47Dco and MCF7 human breast adenocarcinoma cells. mAb 631 and mAb 390 inhibited cell growth by 86 and 68%, respectively, in the estrogen receptor-negative T47Dco cells and by 20 and 71%, respectively, in the estrogen receptor-positive MCF7 cells. Conditioned medium prepared from T47Dco cells was assessed for the presence of Prl-like molecules by its ability to stimulate growth of Prl-responsive Nb2 rat lymphoma cells. Growth of Nb2 cells under the influence of human Prl or conditioned medium was abolished when either solution was pretreated with mAb 390, followed by Immunobead precipitation (Bio-Rad, Melville, NY). T47Dco cells secrete 0.7 μg lactogen/ml over a 24–48-h period. With the use of reverse transcription-PCR, an expected 612-bp band was detected by ethidium bromide staining, and its similarity to pituitary Prl was confirmed by Southern blot analysis with the use of human Prl cDNA as a probe. A single M_r 22,000 band, the dominant size of monomeric pituitary Prl, was found in immunoprecipitates of both cell extracts and conditioned medium from T47Dco cells labeled metabolically with [^{35}S]cysteine. These data suggest that human breast cancer cells synthesize and secrete biologically active Prl.

INTRODUCTION

Endocrine therapy alone or in combination with chemotherapy is a hallmark of breast cancer treatment. This approach is aimed at reducing circulating levels of classical hormones known to stimulate tumor growth or to block their action at the target tissue. The primary target of such therapies to date has been estradiol, long held to be the primary mitogen for human breast cancers. However, the exclusive focus on estradiol may have obscured the role of other hormones and growth factors in this disease.

The development of the hormone-resistant or hormone-independent phenotype, as well as experimental evidence in a variety of model systems, suggests that other hormones such as progesterone and Prl[2] may also play roles in the development and progression of breast cancer. Both progesterone and Prl are known mitogens for normal breast tissue and are involved in rodent mammary tumor development. Prl promotes growth of mammary tumors *in vivo* in rodent models (1). *In vitro*, breast cancer cell growth under the influence of Prl varies depending on the specific experimental conditions used. Conditioned media obtained from Prl-treated nitrosomethyl urea-induced rat mammary tumor cells were able to stimulate colony formation by these same cells grown in soft agar (2). A variety of human breast cancer cell lines responds to the mitogenic signal of Prl *in vitro* under proper conditions of serum staging (3).

Prl receptors (4) and immunologically detectable Prl (5, 6) are present in approximately 70% of human breast biopsy samples, yet clinical evidence for direct effects of Prl on the establishment and/or progression of the disease is lacking. Neither risk nor prognosis has been correlated clearly with circulating levels of Prl (7–9). Treatment with the ergot drug bromocriptine, which suppresses pituitary Prl secretion and, hence, lowers circulating levels of the hormone, was without effect in causing regression in breast cancer patients (10, 11). Operating on the assumption that the lack of effect may have been due to the presence of hGH, which is also a lactogen, Manni *et al.* (12) administered a combination therapy of bromocriptine and a somatostatin analogue to a group of women with advanced breast cancer. Circulating levels of Prl were abolished nearly completely in 8 of 9 patients, whereas hGH levels were suppressed in 7 of 9 patients during treatment. Although overall antitumor effects could not be assessed reliably because the patients entering the study had been pretreated heavily with chemotherapeutic agents, only one patient experienced disease stabilization.

Recent reports have demonstrated extrapituitary transcription of the *Prl* gene. *Prl* gene expression is highly tissue specific and has been shown in the uterus (13), lymphoid cells (14), and T cells (15). Northern analysis and PCR were used to demonstrate Prl message in lactating rat (16), goat, and sheep (17) mammary glands, suggesting local synthesis of Prl. Steinmetz *et al.* (18) showed by *in situ* hybridization that *Prl* gene transcripts are present in secretory mammary epithelial cells in pregnant rats. More recently, the presence of *Prl* gene expression was demonstrated by PCR in some primary human breast carcinomas, suggesting that extrapituitary Prl production occurs more frequently than was thought previously (19). Together, these data suggest that the circulating levels of Prl in serum due to pituitary production of the hormone are irrelevant to breast disease, and the autocrine/paracrine action of Prl in breast tissue needs to be examined more closely. To this end, we provide evidence that Prl is synthesized by human breast cancer cells *in vitro*. The biologically active Prl is then secreted into the medium where it acts in an autocrine manner, stimulating cell growth. This growth can be suppressed by anti-Prl mAbs.

MATERIALS AND METHODS

T47Dco and MCF7 Cell Growth. T47Dco cells, an estrogen receptor-negative clone of the T47D human breast adenocarcinoma cell line (20), were obtained from Dr. Robert Dickson (Georgetown University, Washington, DC). The cells were grown routinely in IMEM (Biofluids, Rockville, MD) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. For individual growth experiments, cells were plated in 6-well culture dishes (35-mm diameter) at a density of 2×10^4 cells/ml, 2 ml/dish. Cells were allowed to attach for 1 day; then media were removed and changed to serum-free conditions with media containing ITS$^+$ (insulin-transferrin-selenium-BSA-linoleic acid culture supplement; Collaborative Research, Bedford, MA). Varying concentrations of anti-Prl mAbs from a starting concentration of 1 mg/ml were added as indicated. mAb 631 and mAb 632 were purchased from Chemicon (Temecula, CA). mAb 390 and mAb 391 were purchased from Bioproducts for Science (Indianapolis, IN). After an additional 3 days in culture, cells were harvested after brief trypsinization and counted in a Coulter counter. Cell viability was >95%. All growth experiments were performed at least three times with duplicate cultures.

MCF7 cells, an estrogen receptor-positive human breast adenocarcinoma cell line (American Type Culture Collection, Rockville, MD), were grown routinely in DMEM supplemented with 5% FBS, 0.1 unit/ml insulin, 100

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[2] The abbreviations used are: Prl, prolactin; hGH, human growth hormone; IMEM, improved MEM; FBS, fetal bovine serum; CSS, charcoal-stripped serum; hPrl, human Prl; poly(A$^+$) RNA, polyadenylated RNA; RT-PCR, reverse transcription-PCR.

units/ml penicillin, and 100 μg/ml streptomycin. For individual experiments, cells were plated at the same density as the T47Dco cells. After attachment for 1 day, media were removed and replaced with DMEM containing 5% CSS with or without varying concentrations of mAb 390 or mAb 631. Cells were harvested and counted as above for the T47Dco cells.

Nb2 Cell Growth. Nb2 cells, a highly Prl-responsive rat lymphoma cell line (originally obtained from Dr. Peter Gout, British Columbia Cancer Agency, Vancouver, B.C., Canada), were grown in suspension in Fischer's leukemia media containing 2-β-mercaptoethanol (10^{-4} M) and supplemented with 10% horse serum, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. For individual experiments, cells were plated in 6-well culture dishes (35-mm diameter) at a density of 2×10^5 cells/ml, 2 ml/dish, in serum-free conditions with the use of ITS$^+$. Varying concentrations of hPrl (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD) or conditioned media were added as indicated. Growth of Nb2 cells is linear in the range of 10–1000 pg/ml Prl and is a standard procedure used to determine Prl levels in serum samples (21). After an additional 3 days in culture, cells were collected by centrifugation and counted in a Coulter counter. Cell viability was >95%. All growth experiments were performed at least three times with duplicate cultures.

Preparation of Conditioned Media. T47Dco cells were plated in T-150 flasks (150 cm^2) in media containing FBS and allowed to grow for 2 days. The medium was then changed to IMEM containing 5% CSS for 1 day. The cells were washed subsequently in PBS before adding ITS$^+$-containing medium and allowed to grow until they were approximately 70% confluent (1–2 additional days). The conditioned medium was collected and concentrated with the use of Macrosep centrifugal concentrators (Filtron, Northborough, MA) according to the manufacturer's instructions. Briefly, the medium was centrifuged through a Mr 300,000 cutoff unit in a Sorvall RC-5 Superspeed refrigerated centrifuge at 5,000 × *g* for 2 h to trap large macromolecules. The filtrate was then centrifuged through a Mr 3000 cutoff unit at 5000 × *g* for 2 h or until the entrapped concentrate was one-tenth the original volume. The concentrated conditioned medium was then desalted by adding conditioned water (deionized, distilled water containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg/ml BSA) to the original volume. The medium was centrifuged again through a Mr 3000 cutoff unit, and the concentrate was collected and frozen at −20°C for later use. Unconditioned medium was prepared in a similar manner from medium never exposed to cells.

Pretreatment and Immunoprecipitation of Conditioned Media. Conditioned medium or hPrl solution (1 μg/μl) was first pretreated with a 1:100 dilution of various anti-Prl mAbs for 2 h at room temperature, followed by immunoprecipitation with anti-mouse IgG Immunobeads (Bio-Rad) for 2 h at 37°C. After centrifugation, the resulting supernatant was added to Nb2 cells for growth studies.

RT-PCR. Poly(A$^+$) RNA was isolated according to Hartmann *et al.* (22). One μg of RNA was used as template for cDNA synthesis with the use of Moloney murine leukemia virus-reverse transcriptase and oligodeoxythymidylic acid (Life Technologies, Gaithersburg, MD) at 37°C for 1 h. The sequences used for the sense and antisense Prl primers have been published previously (19) and were synthesized on a MilliGen 8750 DNA synthesizer (Millipore, Bedford, MA). PCR was carried out with the use of 5 μl of each first strand reaction product containing the cDNA, to which was added 300 ng of each primer, 2.5 units of Taq polymerase, and 0.2 mM dNTPs. Amplification was performed on a Perkin Elmer Cetus (Norwalk, CT) PCR instrument for 30 cycles at 91°C for 1 min, 54°C for 1 min, 72°C for 2 min, and with a final 10-min extension at 72°C.

One-tenth of the PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide. The band of predicted size (612 bp) was confirmed as hPrl by Southern blot analysis with the use of a hPrl cDNA probe (American Type Culture Collection) radiolabeled with [γ-^{32}P]dCTP with the use of a random primer kit (Boehringer Mannheim, Indianapolis, IN).

Metabolic Labeling. T47Dco cells were plated into T-150 flasks under normal growth conditions for 2–3 days or until 70% confluent. The medium was replaced by serum- and cysteine-free IMEM for 1 day. [^{35}S]Cysteine (3 μCi/ml; specific activity, 40–250 mCi/mmol; Amersham, Arlington Heights, IL) was added for 18 h. Medium was collected, concentrated, pretreated with mAb 390, and immunoprecipitated as described earlier. A cell extract was prepared by scraping these same cells into PBS, collecting by centrifugation, and homogenizing in 2 ml of a buffer containing 25 mM Tris (pH 7.4) with 0.1% CHAPS detergent (3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate). The extract was pretreated and immunoprecipitated with the anti-Prl mAb. Samples were run on a 3–27% polyacrylamide gradient gel. After SDS-PAGE, the gel was fixed in methanol:acetic acid, enhanced with Autofluor (National Products, Manville, NJ), and exposed to X-ray film at −80°C. Autoradiograms were obtained in 1–7 days.

RESULTS

Effect of Anti-Prl mAbs on T47Dco Cell Growth. To explore the possibility that Prl is synthesized by human breast cancer cells and acts in an autocrine manner to stimulate cell growth, a panel of anti-Prl mAbs was added to the estrogen receptor-negative T47Dco cells in culture. As shown in Fig. 1*A*, varying degrees of inhibition

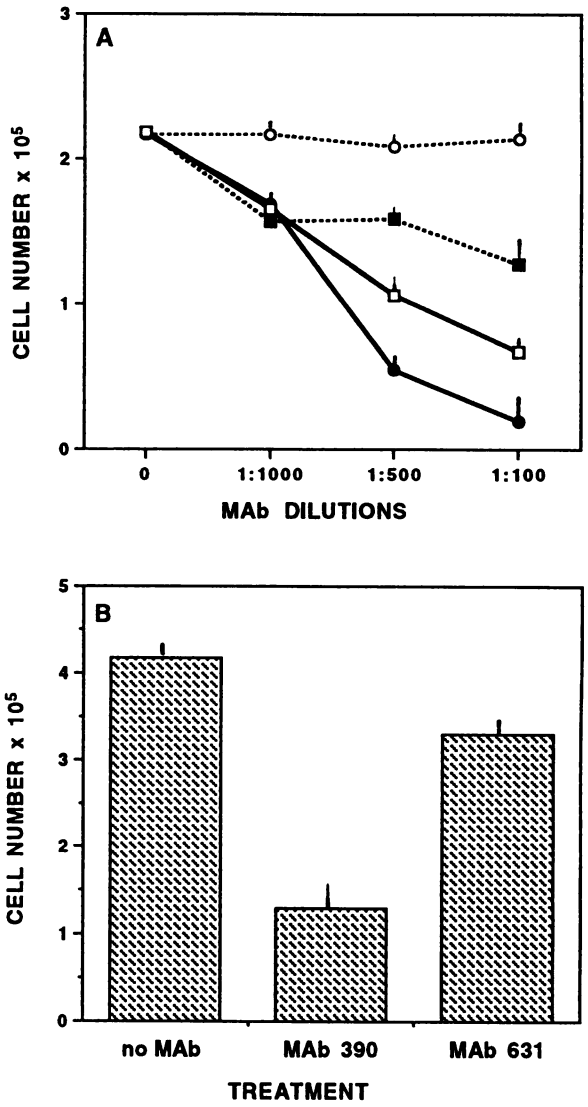

Fig. 1. Effect of various anti-Prl mAbs on T47Dco cell growth. *A*, T47Dco cells were plated in IMEM medium containing 5% FBS in 6-well dishes and allowed to attach for 1 day. The medium was then replaced with serum-free medium. Varying concentrations of anti-Prl mAbs from a starting concentration of 1 mg/ml were added, and the cells were allowed to grow for 3 additional days. After brief trypsinization, cells were counted in a Coulter counter. *Points*, average of 3 experiments with the use of duplicate cultures; *bars*, SE. □, mAb 390; ■, mAb 391; ●, mAb 631; ○, mAb 632. *B*, MCF7 cells were plated in DMEM medium containing 5% FBS in 6-well dishes and allowed to attach for 1 day. The medium was then removed and replaced with medium containing 5% CSS in the presence or absence of 1:100 dilution of mAb 390 or mAb 631. After an additional 3 days, cells were trypsinized and counted in a Coulter counter. *Columns*, average of 2 experiments with the use of duplicate cultures; *bars*, SE.

were achieved. Maximal inhibition was seen at a 1:100 dilution of antibody, with mAb 631 and mAb 390 exhibiting the best overall inhibition (86 and 68% inhibition of cell growth, respectively). Under similar growth conditions, the estrogen receptor-positive MCF7 cells were inhibited by mAb 631 and mAb 390 at a 1:100 dilution by 18 and 71%, respectively (Fig. 1*B*). Therefore, these two antibodies were used in subsequent experiments.

Conditioned Media Stimulate Growth of Nb2 Cells. To determine whether the inhibition by the anti-Prl mAbs was due to neutralizing a secreted Prl-like molecule, conditioned media were prepared from T47Dco cells and added to Prl-responsive Nb2 rat lymphoma cells under serum-free conditions. These cells respond to pg quantities of human pituitary Prl with a 3-fold increase in cell number in 3 days. The Prl-stimulated growth of Nb2 cells was abolished when the hormone was removed from solution by pretreatment with mAb 390, followed by Immunobead precipitation (data not shown). Under similar growth conditions, the Nb2 cells were stimulated nearly 3-fold by concentrated conditioned media from T47Dco cells (Fig. 2). The level of activity in the conditioned media was equivalent to 0.7 μg/ml (14.5 pg Prl/cell) of pituitary Prl and was 3.6% of the amount normally produced by the rat pituitary cell line, GH3 (21, 22). This activity in the conditioned media, like that of the human pituitary Prl, was abolished when the media were pretreated with mAb 390, as well as mAb 631, followed by Immunobead precipitation. Concentrated, unconditioned medium alone was unable to stimulate Nb2 cell growth and did not enhance Prl-stimulated growth. These data indicate that the secreted factor in conditioned medium is a lactogen.

Detection of Prl Transcripts by RT-PCR. The presence of Prl-like activity in conditioned media from T47Dco cells suggested that human breast cancer cells in culture synthesize and secrete Prl. Therefore, RT-PCR was utilized to look for the message for Prl in T47Dco and MCF7 cells. The RT-PCR reactions with the use of poly(A^+) RNA prepared from these cells (Fig. 3, *Lanes 3* and *5*) produced an expected 612-bp band by ethidium bromide staining. That this product is related to pituitary Prl was confirmed by Southern analysis (Fig. 3) with the use of hPrl cDNA as the probe. PCR of the hPrl cDNA (Fig. 3, *Lane 2*), used as a positive control, also gave a band at 612 bp; poly(A^+) RNA from lactating mouse mammary glands (Fig. 3, *Lane 4*) acted as a negative control because there is <70% homology between human and mouse Prl sequences.

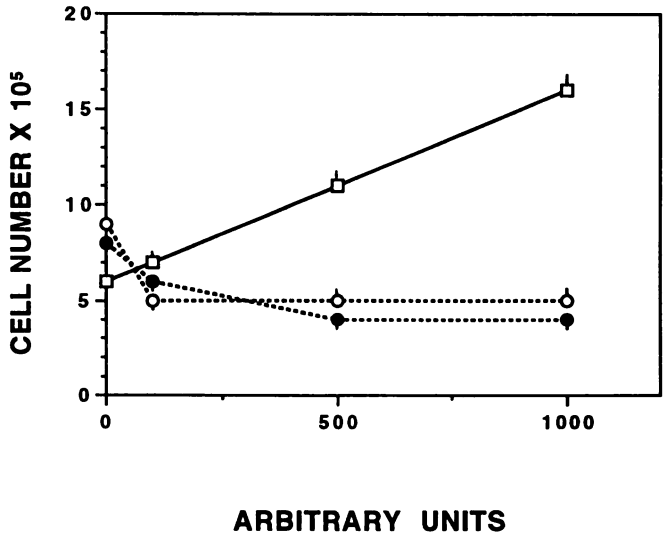

Fig. 2. Prl-like activity in conditioned medium. Nb2 cells were plated in serum-free Fischer's leukemia medium with varying amounts of conditioned medium and allowed to grow for 3 days. Cells were counted in a Coulter counter. *Points*, average of 3 experiments with the use of duplicate cultures; *bars*, SE. Nb2 cell growth with the use of conditioned medium alone (□) or conditioned medium pretreated with mAb 390 (●) or mAb 631 (○).

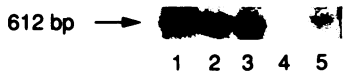

Fig. 3. Detection of Prl by Southern blot analysis after RT-PCR. Autoradiogram was obtained after 4 days. *Lane 1*, 600-bp marker of an ethidium bromide-stained 100-bp DNA ladder; *Lane 2*, hPrl cDNA; *Lane 3*, MCF7 cells; *Lane 4*, lactating mouse mammary gland; *Lane 5*, T47Dco cells.

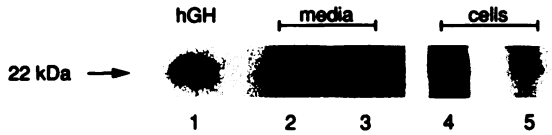

Fig. 4. Detection of M_r 22,000 protein in conditioned media and cells after metabolic labeling. T47Dco cells were labeled metabolically with [^{35}S]cysteine for 18 h. Media and cells were collected and prepared, pretreated with or without mAb 390 and immunoprecipitated, and run on a 3–27% polyacrylamide gradient gel as described in "Materials and Methods." *kDa*, molecular weight in thousands. *Lane 1*, ^{125}I-labeled hGH (30-min exposure); *Lane 2*, conditioned medium treated with mAb 390 (1-week exposure); *Lane 3*, conditioned medium without mAb 390 (1-week exposure); *Lane 4*, cell extract treated with mAb 390 (1-day exposure); *Lane 5*, cell extract without mAb 390 (1-day exposure).

Metabolic Labeling of T47Dco Cells. Since our data indicated that human breast cancer cells contained message for a Prl-like molecule with biological activity, its physical relationship to pituitary Prl was examined by metabolically labeling T47Dco cells with [^{35}S]cysteine. Conditioned media and cell extracts were pretreated in the presence or absence of mAb 390 as described in "Materials and Methods," collected by Immunobeads, and run on a 3–27% polyacrylamide gradient gel. As shown in Fig. 4, a band of M_r ~22,000, the size of pituitary Prl and hGH, was present when conditioned medium (Fig. 4, *Lane 2*) or cell extract (Fig. 4, *Lane 4*) was precipitated by the anti-Prl mAb. No band was seen when the mAb was excluded from the immunoprecipitation reactions (Fig. 4, *Lanes 3* and *5*). The presence of the M_r ~22,000 band in the cell extract, as well as in the conditioned medium, suggests that human breast cancer cells both synthesize and secrete hPrl.

DISCUSSION

One of the most puzzling questions in mammary gland biology has been why Prl, which clearly plays an important role in the induction and progression of mammary tumors in rodents (1), has not been defined as clearly as a critical player in human breast cancer. *In vitro*, primary cultures of human mammary epithelial cells (23), like their rodent counterparts (24, 25), display an absolute requirement for Prl for growth and passage on tissue culture plastic or inside of collagen gels. Investigations using human breast tumor tissue and cells show clear responses to lactogenic hormones. These responses include increased DNA (26–29), protein (30), and α-lactalbumin (31) synthesis; colony formation (23, 32); changes in shape, adhesion and lipid accumulation (33); and estrogen receptor content (34). In addition, we have shown that most human breast cancer cell lines express Prl receptors and are induced by Prl to grow when impeding bovine lactogens are first removed from the fetal bovine serum in which they are grown routinely (3, 35). From 60–85% of human breast cancer biopsies contain immunologically detectable Prl (5, 6), whereas specific Prl receptors have been demonstrated in over 70% of the biopsy samples (4, 36–38).

Over the years, significant progress has been made in defining the role of estrogens in human breast cancer because removing the primary source of estrogens (*i.e.*, the ovaries) or inhibiting estrogen action at the target tissue with antiestrogens such as tamoxifen has

altered clearly the course of the disease. Similar studies with pituitary Prl have been discouraging or inconclusive. There is no clear correlation between circulating Prl levels and the etiology or prognosis of the disease (7–9). When patients are treated with Prl-inhibiting ergot drugs, which effectively eliminate circulating pituitary Prl, no change in disease state is reported (10, 11). These data point strongly to a need for an alternate approach to the question of the role of Prl in human breast cancer.

Recent reports concerning expression of Prl mRNA in breast tissue from a variety of species, as well as the data presented herein, suggest that it is an autocrine rather than an endocrine Prl that may function in breast cancer. LeProvost *et al.* (17) have shown by Northern analysis and RT-PCR that mammary glands from late pregnant and lactating sheep and goats express the *Prl* gene. Sequence analysis of the cDNA generated from these tissues differed from pituitary transcripts by only 3 mutations, 2 of which were silent. Unlike the *Prl* gene in placenta (39) and lymphocytes (15), the mammary gland gene appears to be transcribed from the same promoter as in the pituitary (17). This similarity between pituitary and mammary gland Prl at the level of the gene suggests that posttranslational events may be important to autocrine function of Prl.

We have shown that in addition to mRNA for Prl, the M_r 22,000 Prl protein product is also synthesized and secreted by human breast cancer cells. On the basis of biological activity on Nb2 cells, the conditioned medium from T47Dco cells grown under serum-free conditions contains significant amounts of Prl. It is clear that multiple forms of Prl are found in tissues and biological fluids from a variety of species including humans (40–43). These variants include Prls of several sizes, as well as posttranslational modifications. The diversity of actions of Prl may be attributed to the variants that change in abundance with the physiological state of the animal (42). It is now clear that Prl, which is detected in milk with the use of RIAs developed for pituitary Prl, may not be solely of pituitary origin. Posttranslational modification of Prl results in variant forms that display biological activity and immunoreactivity different from dominant, monomeric M_r 22,000 pituitary Prl (40, 41). In this study, by metabolic labeling of the T47Dco cells, we were able to detect only one size variant of Prl, *i.e.*, M_r 22,000. Thus, any regulation of secretion or biological activity of mammary-derived Prl most likely would be posttranslational. The variation in response obtained with different mAbs prepared against pituitary Prl, which we used in this study, may reflect the diversity of posttranslational modifications to mammary-derived Prl. Biological activity and immunoreactivity of Prl are influenced by phosphorylation and glycosylation of the Prl variants. Differences in the oligosaccharides attached to Prl alter biological activity (44, 45). Dephosphorylation of pituitary Prl increases its bioactivity in the Nb2 assay by 2-fold (43). The regulatory mechanisms involved in the posttranslational modification of Prl variants are unknown.

Interaction of Prl with its receptors is a necessary first step in its biological action. The existence of several forms of the receptor and their location in the nucleus and on internal membranes as well as the cell surface (46) present a variety of possibilities for the mode of action of endogenous Prl. We have demonstrated that breast cancer cells synthesize and secrete the hormone, suggesting an autocrine mechanism. However, an intracrine mechanism cannot be ruled out at this time. The ultimate fate of the hormone-receptor complex as it sets into motion the cascade of events leading to mitosis remains to be elucidated.

Whether other hormones and growth factors that influence growth of human breast cancer cells in culture, such as estrogens, progesterone and EGF, also affect the synthesis and secretion of the various biologically active forms of Prl is currently under study. Similarly, whether the underlying synthesis of Prl is essential for the growth-promoting activity of these other agents needs to be explored. Insights into these hormone and growth factor interactions and the significance of the autocrine role of Prl in human breast cancer may have considerable impact on treatment strategies in the future.

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