Evidence for a Novel Pituitary Factor That Potentiates the Mitogenic Effect of Estrogen in Human Breast Cancer Cells

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ABSTRACT

Estrogen, prolactin, and other tissue-derived factors are implicated in the etiology and pathophysiology of human breast cancer (HBC). In a previous study, we demonstrated that a factor(s) secreted by rat pituitary tumor cells (GH3) synergizes with estrogen to induce growth of HBC cells (T-47D) transplanted into athymic nude mice. The present studies were carried out to characterize further this pituitary growth factor. Pituitary tumor cell lines (GH3, GH1, 235-1, and AtT-20) and normal rat pituitaries were transplanted s.c. into estrogen-treated (estradiol valerate injection, 500 µg/14 days) athymic nude mice which also received T-47D cells. The influence of the presence of these normal and tumorous pituitary cells on growth (size and weight) of T-47D tumors was monitored for 49 to 56 days. The results indicate that factor(s) from normal rat pituitary glands as well as from the GH3 and GH1 but not 235-1 and AtT-20 pituitary tumor cells were able to potentiate the growth of T-47D tumors in estrogenized mice. To ascertain whether or not prolactin and/or growth hormone are responsible for the growth-promoting activity, purified human and ovine growth hormone and ovine prolactin were administered to estrogenized athymic nude mice either by daily s.c. injection (100 µg/day) or by constant infusion using Alzet osmotic minipumps (1.25 and 5.0 µg/h) for 49 to 56 days. None of these treatments stimulated the growth of the T-47D tumors, suggesting that prolactin, growth hormone, and their intermediates may not be directly involved. We further determined whether the factor from pituitary tumor cells was present in serum-free conditioned medium and could stimulate the growth of HBC cells in vitro. Conditioned medium from GH3 and GH1, but not from 235-1 and AtT-20 pituitary cells significantly stimulated growth of T-47D cells in the presence of estradiol (10^{-10} M) after 12 days of culture in a serum-free medium (Dulbecco's modified Eagle's medium containing bovine serum albumin, 0.5 mg/ml). Optimal serum-free growth of T-47D cells (2-fold above control) was observed in the presence of estradiol (10^{-10} M) and conditioned medium (30% v/v) from 48-h cultures of GH3 cells. The bovine serum albumin concentration of the serum-free medium (Dulbecco's modified Eagle's medium) was also important: optimal T-47D cell proliferation was observed with BSA between 0.5 and 2.0 mg/ml. Conditioned medium preparations from serum-pre-treated flasks (without cells) from GH3 cell monolayers for zero time and from actinomycin D plus cycloheximide-inhibited GH3 cells were inactive. In addition, the GH3 pituitary factor potentiated the estrogen effect only in those HBC cells that contained estrogen receptors (T-47D, T-47D clone 11, MCF-7, ZR-75-1);

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The present studies were carried out to further characterize this pituitary growth factor for breast cancer. We now report that: (a) growth of T-47D tumors in estrogenized athymic nude mice were stimulated by factor(s) from both transplanted normal pituitary glands and certain tumorous pituitary cells; (b) long-term administration of highly purified prolactin and growth hormone to estrogenized athymic nude mice did not potentiate the mitogenic effect of estrogen; (c) CM prepared from rat pituitary tumor cells (GH3 and GH1) stimulated T-47D cells in a serum-free medium in the presence of estrogen; little growth-promoting activity was observed in the absence of steroid; and (d) GH3 CM was found to potentiate the estrogen-induced growth of those
HBC cell lines that contained estrogen receptors; estrogen receptor-negative tumor cells were not responsive to estrogen whether or not the pituitary factor was present. These results support our hypothesis that a novel pituitary growth factor for estrogen receptor-positive HBC which is distinct from all known pituitary hormones may exist.

MATERIALS AND METHODS

Tumor Cell Lines. Eight human breast-derived and 4 pituitary tumor cell lines were used in these studies. The rat pituitary tumor-derived cell lines GHt, and its clonal variant GH2, and the mouse pituitary tumor line AtT-20 were obtained from the American Type Culture Collection, Rockville, MD. The rat pituitary clonal cell line, 235-1 was kindly provided by Dr. M. Reymond, University of Texas Southwestern Medical School, Dallas, TX. The HBC cell lines, T-47D, BT-474, BT-20, MDA-MB-231, and the nonpituitary tumor cell line HBL-100 were obtained from EG and G Mason Research Institute, Rockville, MD.; the subline (clone 11) of the T-47D cell line was kindly provided by Dr. I. Keydar, University of Tel Aviv; MCF-7 cells were kindly supplied by Dr. M. Rich, formerly of the Michigan Cancer Foundation, Detroit, MI; and ZR-75-1 cells were kindly supplied by Dr. M. Lipman, National Cancer Institute, Bethesda, MD. All cell lines were routinely maintained in DMEM supplemented with L-glutamine (4 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% (v/v) FCS. Trypsin-EDTA in Hanks' balanced salt solution was used for cell passages at weekly intervals. All the above reagents were purchased from Grand Island Biological Co., Burlington, Ontario. Cells were maintained in a humidified atmosphere of 95% air:5% CO2 at 37°C.

Animals. Four- to 5-week-old female BALB/c athymic nude mice were obtained from ARS/Sprague-Dawley Division, Madison, WI. Animals were kept under standard conditions for a 5- to 7-day period prior to use. The animals were kept inside a laminar-flow air filtration system, and feed and water were supplied ad libitum.

Hormones. Purified ovine growth hormone (NIH-GH-S11) and prolactin (NIAMDD-o-PRL-15) were obtained from the National Pituitary Agency, Baltimore, MD. Purified human growth hormone was provided by Dr. H. G. Friesen, University of Manitoba. Long-acting estradiol valerate was the product of Squibb Canada Inc. Estradiol was obtained from Sigma Chemical Co.

Inoculation of Cells and Growth of Tumors in Nude Mice. Cells were detached with trypsin-EDTA in Hanks' balanced salt solution and resuspended in a small volume (approximately, 1 ml) of growth medium (DMEM). The cells were injected s.c. in the flanks of the animals, T-47D and other HBC cell lines were plated in 35-mm dishes at cell densities of 10^2 to 10^5 cells/dish, as indicated in the legends to Charts 3 to 8, in DMEM with glutamine, antibiotics, and 10% FCS. Cells were incubated for 48 h to allow for cell attachment. The medium was then discarded and replaced with DM-500 (2 ml). After 24 h, this medium was replaced by fresh DM-500 or DM-500 containing estradiol 10^-10 M. Test substances (pituitary cell CMs) were then added at the indicated concentrations to HBC cells growing in the absence or presence of estradiol. After 12 days of culture, a medium change on Day 6, cells were detached with trypsin-EDTA, dispersed in isoton (Fisher Scientific Co.), and counted in a Coulter Counter. For each determination, triplicate dishes were used.

Statistics. Differences between 2 groups were analyzed using Student's t test for 2 independent samples. Analysis of variance was used when comparing multiple groups.

RESULTS

Tumor Growth of T-47D Cells in Athymic Nude Mice. In a previous study, we showed that the growth in vivo of T-47D human breast tumor cells in athymic nude mice required estrogen, and more importantly, transplantation of pituitary tumor cells (GH2) potentiated the effect of estrogen (17). It was uncertain, however, from this study as to the identity of the pituitary hormone involved.

In the present studies, we determined the specificity of this effect and hence whether other pituitary tumor cell lines and normal pituitary glands were also active in stimulating the growth of T-47D HBC cells in the presence of estrogen (Charts 1 and 2). Transplantation of GH1 and GH3 cells significantly enhanced the growth of T-47D tumors after 49 days; the mean tumor size of the GH1 group, 465 ± 82 (SD) cu mm, and GH3 group, 350 ± 54 cu mm, represented 4- and 3-fold increases in T-47D tumor size as compared to that of the estrogen only control group (124 ± 12 cu mm) (Chart 1A). The mean T-47D tumor size in the AtT-20 group was 165 ± 42 cm mm which was not significantly different from the control. These effects appeared to require the higher dose of estrogen (500 µg/14 days) administered from Day 28, as evidenced from the lack of early response to the presence of GH1 and GH3 cells with lower-dose estradiol (170 µg/14 days) given from the start of the experiment (Chart 1A). Similarly to the AtT-20 line, 235-1 cells were also unable to cause synergistic growth effects with estrogen on T-47D tumors (Chart 1B). Transplantation of normal rat pituitary glands instead of the rapidly proliferating pituitary tumor cell lines, in experiments similar to those described in Chart 1, were also capable of enhancing T-47D tumor growth in the presence of estrogen (Chart 2). Accumulated data from several experiments as indicated showed that normal rat pituitaries and pituitary tumor cells (GH1 and GH2) produced similar growth of T-47D tumor in the presence of estrogen. These results indicate that factor(s) from normal rat pituitary glands as well as from some pituitary tumor cell lines are able to stimulate the growth of T-47D tumors. To ascertain whether or not prolactin and/or growth hormone are directly...
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Chart 1. Effects of estrogen and pituitary tumor cells on growth of T-47D tumors in nude mice. A. beginning of experiment; all animals received lower dose of estradiol valerate (E2; 170 µg/14 days), T-47D, and pituitary cells as indicated; from Day 28, a higher dose of estradiol valerate (500 µg/14 days) was substituted for lower dose. All points, mean ± SD (bars) for 4 animals. B. similar protocol as above, except that estradiol valerate was administered at 500 µg from time zero. Procedures for determining growth of tumors are described in "Materials and Methods."

responsible for the growth-promoting activity, purified ovine prolactin, ovine growth hormone, and human growth hormone were administered into estrogenized athymic nude mice either by osmotic minipumps or by daily s.c. injection. Chart 2 shows that none of these treatments significantly stimulated the growth of T-47D tumors. T-47D tumor weights determined from sacrificed animals at the end of the experiments were in good agreement with final tumor size measurements from all experimental groups.

The results of Charts 1 and 2 indicate that prolactin and growth hormone, the principle hormones known to be produced by GH3 and GH4 pituitary tumor cells and by normal pituitary glands removed from hypothalamic control, are not responsible for potentiating the estrogen effect on T-47D tumor growth. These studies also suggest that a novel pituitary factor could be responsible for potentiating the mitogenic effect of estrogen in T-47D HBC cells.

In Vitro Studies. If GH3 pituitary tumor cells secrete a factor that stimulated the T-47D tumor in estrogenized nude mice, we may expect that this factor would also be secreted into the culture medium of GH3 cells. Chart 3 shows that the estrogen-requiring growth-promoting activity for T-47D cells in vitro was indeed present in CM from GH3 cells. This effect was dependent on the time of conditioning of the GH3 tumor cells; the most significant growth response on T-47D cells was observed using GH3 CM collected at 48 h. Under our assay conditions using serum-free medium, GH3 CM alone (in the absence of estrogen) produced relatively little growth of T-47D cells. It is somewhat puzzling, however, that a 64-h CM significantly inhibited the estrogen-requiring growth (observed in 2 experiments) when compared to the 48-h CM. Such an effect may suggest that the pituitary factor is labile or that an inhibitor was accumulated. CM from 48-h cultures of GH3 cells (stimulated growth, 170% of control; Chart 3) was used subsequently in further experiments.

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The in vitro assay was further optimized with respect to the concentration of GH3 CM which gave maximum growth of T-47D cells in the presence of estradiol. Optimal responses were observed between 27 and 33% (v/v) (Chart 4); 30% CM was used subsequently in further studies.

The results presented in Chart 5 show the time course of T-47D cell growth in response to CM and estradiol. Untreated T-47D cells grew slowly in the serum-free medium (DM-500) with a doubling time of approximately 6 days. The addition of either estradiol (10^{-10} M) or 48-h GH3 CM to T-47D cultures resulted in similar enhanced growth rates for both conditions (doubling time of 3 days), while cells treated simultaneously with CM and estrogen doubled their cell number within 2 days. In this series of optimization experiments in the development of an in vitro assay for this pituitary factor, the concentration of BSA in DMEM was varied. The GH3 CM was unable to stimulate growth of estrogenized T-47D cells at low BSA concentration (below 0.2 mg/ml). BSA concentration at 0.5 mg/ml was found to be optimal (Chart 6).

Four types of control conditions for the preparation of pituitary tumor cell CM were studied to determine whether the growth-promoting activity of GH3 CM was contributed from residual serum contamination. Chart 7 compares the growth of T-47D HBC cells in (a) unconditioned DM-500, (b) 48-h CM of GH3 cells treated previously with actinomycin D (ACT.D) (1 ug/ml) and cycloheximide (CHX) (10 mw) in 10% FCS medium for 24 h, 48-h CM of flasks without GH3 cells, exposed previously to 10% FCS medium for 24 h (EMPTY FLASK); CM in contact with GH3 cells for zero time (CM, 0-HR) and 48 h CM of pituitary cell lines indicated. All CMs were added at 30% (v/v) to dishes of cells treated with or without estrogen. Cell growth in the absence of estrogen is shown as 100% (C), and the percentage increase (%) represents estradiol-potentiating growth activity observed for the respective conditions after 12 days. Numbers of experiments are indicated; bars, mean ± SE. *P< 0.001 compared to other (nonasterisked) conditions.

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enhanced T-47D tumor growth in the presence of estrogen by cated in the proliferation of HBC cells in estrogenized athymic this phenomenon and the putative pituitary growth factor impli
ation of HBC cells.

for and identify other factors which might regulate the prolifera
lack of convincing evidence for a role of prolactin in the prolifer
(c) cell lines derived from neoplastia tissue frequently synthesize
many growth factors other than the traditional hormones; and
increasing evidence to suggest that the pituitary gland produces
these hormones is influenced by estrogen (20); (D) there is ever
use GH3 cells in these experiments since: (a) they are known to
athymic nude mice is stimulated by the simultaneous presence
of estrogen on T-47D cells in contrast to the low levels of activity present in CM of 235-1 and AtT-20 cells. Finally, a total of 7 HBC cell lines and one nontumor breast milk-derived cell line were studied to determine if the pituitary factor in GH3 CM demonstrated any specificity towards its target cells (Chart 8). CM of GH3 pituitary tumor cells was able to potentiate the mitogenic effect of estrogen only in those HBC cells that contained estrogen receptors, namely, T-47D, MCF-7, T-47D clone 11, and ZR-75-1 lines. Estrogen receptor-negative tumor cells BT-22, MDA-MB-231, and BT-20 and the nontumor mammary cell line HBL-100 were not responsive to estradiol treatment, whether or not the GH3 CM was present.

DISCUSSION

The results of our previous in vivo study showed that the growth of human breast cancer cells (T-47D) transplanted into athymic nude mice is stimulated by the simultaneous presence of estrogen and GH3 rat pituitary tumor cells (17). We chose to use GH3 cells in these experiments since: (a) they are known to secrete prolactin and growth hormone, and the secretion of these hormones is influenced by estrogen (20); (b) there is ever increasing evidence to suggest that the pituitary gland produces many growth factors other than the traditional hormones; and (c) cell lines derived from neoplastic tissue frequently synthesize biologically active products in increased amounts. In view of the lack of convincing evidence for a role of prolactin in the proliferation of human breast cancers (4), there is more reason to search for and identify other factors which might regulate the proliferation of HBC cells.

The present studies were undertaken to further characterize this phenomenon and the putative pituitary growth factor implicated in the proliferation of HBC cells in estrogenized athymic nude mice as observed previously (17). The phenomenon of enhanced T-47D tumor growth in the presence of estrogen by pituitary tumor was not confined to the GH3 cell line but was shared by other prolactin- and growth hormone-secreting cells, namely, GH1 rat pituitary tumor cells and normal rat pituitary glands (Chart 2). Russo et al. (21) had demonstrated previously that MCF-7 HBC cells formed tumors in nude mice that received transplants of normal pituitary glands and ovarian grafts suggesting that the normal pituitary gland is capable of producing growth factors which stimulate proliferation of HBC cells. Our studies suggest that similar factors are present and secreted from both normal and tumorous pituitary cells and that these are responsible for the augmentation of T-47D tumor growth in the estrogenized nude mouse. The permissive role of the pituitary is not confined to the GH3 system. A potentially similar pituitary factor(s) is also strongly implicated in the induction and growth of estrogen-dependent renal adenocarcinomas (22) and in the inhibition of ovariectomy-induced rat mammary tumor regression (23).

In our previous study (17), it was somewhat surprising to observe that in mice bearing GH3 tumors, no apparent growth of the HBC cells was observed in the absence of estrogen despite unphysiologically high prolactin and growth hormone concentrations in the blood of the host. Thus, clearly rat prolactin and/or growth hormone alone were not sufficient to stimulate the proliferation of T-47D tumor in nude mice. Nevertheless, to ascertain whether or not "higher" prolactins and/or growth hormones could be at least in part responsible for the growth promoting activity, highly purified human growth hormone, ovine prolactin, and ovine growth hormone were administered into estrogenized athymic nude mice. None of these treatments stimulated the growth of the T-47D tumors, confirming that prolactin, growth hormone, and their intermediates were not directly involved. These studies, however, do not preclude a permissive role for prolactin in human breast cancer. Furthermore, our experiments support the current view that estrogen is involved in the growth regulation of some HBCC. However, injection of estrogen alone resulted only in a very moderate growth of T-47D tumor. This suggests that estrogen alone cannot produce maximal growth of the T-47D tumor. Other hormones, factors, or mediators are required. Clearly, the in vivo animal model is extremely interesting but undoubtedly complex. Several mechanisms therefore had to be proposed previously to account for growth stimulation of human breast tumors in estrogenized animals transplanted with pituitary cells (17, 18).

Growth of the human breast cancer cell line, T-47D, has been shown to be responsive to estrogen both in vivo (17) and in vitro (24). These observations prompted an attempt to develop an in vitro assay for the detection of the putative pituitary factor; CM of pituitary tumor cells from GH3 and GH1 was indeed able to potentiate the mitogenic effect of estrogen on HBC cells in the absence of serum. Our in vitro findings could at least in part explain our findings in vivo. There is no need therefore to invoke the presence of estrogen-induced host growth factors (estromedins) (12).

In other studies, prolactin and growth hormone by themselves or in combination with estrogen were unable to stimulate the growth of T-47D cells in vivo (25). In fact, no convincing direct mitogenic effects of prolactin and growth hormone on HBC cells in long-term culture have been reported (26, 27), although a
recent report showed that proliferation of early passage cells in culture established from a human metastasizing carcinoma was increased in the presence of prolactin (28). Whether prolactin in general is directly mitogenic in the early stages of endocrine-responsive primary breast tumor development and secondary metastasis is, however, unknown.

It appears that the in vivo effects of pituitary factor and estrogen were synergistic. In vitro, both synergism (e.g., Chart 6) and additive effects (e.g., Chart 5) of pituitary factor and estrogen were observed. Also, the magnitude of in vivo effect of pituitary factor was greater than that in vitro. Several reasons may account for these differences. Pituitary tumors growing in athymic mice were able to deliver a constant supply of growth factor to the T-47D tumor; in vitro studies with T-47D cells were dependent on replenishment of pituitary growth factor and estrogen and hence limited by the frequency of refedding of cultures. Furthermore, the duration of in vivo and in vitro experiments differed; the studies with athymic nude mice lasted between 49 and 56 days, while serum-free culture experiments were no longer than 12 days.

Our pituitary-derived growth factor appears to be distinct from all known pituitary hormones and is therefore a potentially novel pituitary peptide "hormone." Our results and conclusion are similar to that of 2 clinical studies during 1959 to 1960 which showed that estrogen but not prolactin or growth hormone injected into breast cancer patients stimulated the growth of the tumors, whereas it failed to do so when injected into the same patients after hypophysectomy (29, 30). Indeed, the authors of these clinical studies suggested that a pituitary factor was required for estrogen action in breast cancer patients. In addition, our laboratory (Charts 7 and 8; Ref. 31) and other workers (32, 33) over the last few years have observed that although estrogen stimulates the growth of breast cancer in vivo, the steroid hormone has no or little mitogenic effect on the cancer cells in vitro (in cell culture) unless steroid-depleted fetal bovine serum is used. This in vitro observation suggests that serum contains a protein factor(s) that is required for estrogen to manifest fully its effect. We therefore speculate that the factor present in serum may be similar to the factor produced by the pituitary gland. Furthermore, addition of pituitary extract to a hormone-supplemented defined medium is mandatory to support optimal growth of normal human mammary epithelial cells and results in their rapid clonal proliferation and extended serial passage in culture, clearly implying that undefined pituitary factors are also important for sustained growth of normal mammary cells (34).

The simplest mechanism therefore which can account for both in vivo and in vitro findings is that the pituitary cells produce a growth factor (which is not prolactin or growth hormone) which stimulates the growth of T-47D cells, provided that estrogen is present. This factor potentiates the estrogen effect only in estrogen receptor-positive HBC cell lines; estrogen receptor-negative tumor cells do not respond to estrogen whether or not the pituitary factor is present (Chart 8). At present, the mechanism by which the pituitary factor potentiates the estrogen-dependent growth of HBC cells is not known.

Finally, whether or not the pituitary factor resembles other reported growth factors such as tumor growth factor or insulin-like growth factor remains to be determined.

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