Gonadotropin-releasing Hormone Gene Expression in MDA-MB-231 and ZR-75-1 Breast Carcinoma Cell Lines

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ABSTRACT

The presence of gonadotropin-releasing hormone (GnRH)-binding sites in human breast carcinomas and breast tumor cell lines as well as the demonstration of the inhibitory effects of GnRH analogues on the growth of these cells raised the possibility that GnRH is produced locally by breast tumor cells themselves. Immunoreactive GnRH was shown to be present in acetic acid extracts of cultured MDA-MB-231 and ZR-75-1 breast carcinoma cells. These extracts were separated by high-performance liquid chromatography and were analyzed by means of region-specific antisera with differing GnRH sequence specificities. A peak of GnRH which coeluted with synthetic mammalian GnRH in 2 different high-performance liquid chromatography systems was similarly detected by antisera directed at the NH₂ terminus, at the middle portion and at the N and COOH termini together. The GnRH gene is expressed in these breast tumor lines, as determined by Si nuclease protection assay, oligonucleotide primer extension studies, and polymerase chain reaction amplification of complementary DNA using oligonucleotides. The primer extension studies indicate that several forms of mRNA are present. The predominant form corresponds to the excision of intron I and the use of a start site about 60 bases upstream of intron I as in the human hypothalamus. Less usage is made of other start sites further upstream. Much larger species of mRNA were also present and correspond to the retention of intron I as in human placenta. The demonstration of GnRH gene expression and the presence of immunoreactive GnRH in mammary carcinoma cells known to have GnRH-binding sites and to be affected by GnRH analogues suggests that GnRH may serve an autocrine regulatory role.

INTRODUCTION

In mammals, pulsatile administration of physiological concentrations of the hypothalamic peptide, GnRH¹ (1, 2), stimulates the secretion of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. However, high doses of GnRH and GnRH agonists or continuous infusion of these peptides induce pituitary desensitization with a subsequent reduction in gonadotropin secretion and diminished plasma levels of gonadal steroids (3). In addition, prolonged administration of GnRH results in altered posttranslational processing of gonadotropin to forms with low biological activity. GnRH agonists have also been reported to have a direct inhibitory action on gonadal steroidogenesis in animal models (4-6).

Superactive GnRH agonists have been used successfully in the treatment of patients with hormone-dependent mammary and prostatic carcinomas (7-9). This action is presumed to result from a lowering of plasma levels of gonadal steroids (10). However, certain postmenopausal women with mammary carcinomas have responded to GnRH agonist therapy (11, 12).

Because postmenopausal women have low ovarian steroid hormone production, it was proposed that GnRH might have a direct action on mammary tumors (13). The presence of specific GnRH-binding sites (Ka 10⁻⁴ M) in human breast carcinomas, but not in normal breast tissue, provided a means for this effect (13). In order to further explore the significance of this finding, we screened a number of breast carcinoma cell lines for the presence of GnRH-binding sites. Specific GnRH-binding sites were detected on cultured human breast carcinomas and had an apparent Ka on the order of 10⁻⁴ M (14, 15).

Support for the direct action of GnRH arose from observations that GnRH analogues inhibited both thymidine incorporation into DNA and proliferation of breast tumor cell lines (14, 15). Since GnRH is undetectable or very low in the general circulation, these findings suggested the possibility that GnRH might be produced locally within breast carcinomas. We have therefore examined two human breast carcinoma cell lines for the presence of GnRH peptide and mRNA. Both ZR-75-1 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) have GnRH-binding sites and respond to GnRH analogues (15).

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma cell lines MDA-MB-231 and ZR-75-1 cells were gifts from J. L. Wittliff, University of Louisville (Louisville, KY). The cells were grown at 37°C in Dulbecco's modified Eagle's medium (Flow Laboratories, Ayrshire, Scotland) containing 10% fetal calf serum in 75- or 150-cm² flasks. The cells were checked for Mycoplasma contamination using the DNA fluorochrome Hoechst 33258 (Flow Laboratories).

Peptide Extraction from Cells. Cells were grown in 150-cm² flasks until they had reached confluence and were then harvested by incubation for 15 min with PBS, pH 7.4 (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ · 2H₂O, and 0.2 g KH₂PO₄/liter) containing 0.6 mM EDTA. The cells were washed twice in PBS, and the cell pellets were subsequently disrupted at 4°C with a glass Tenbroeck homogenizer in 2 mM acetic acid containing 1 mM phenylmethylsulfon fluoride. The extracts were clarified by centrifugation at 12,000 x g for 45 min, and the supernatants were twice lyophilized. These lyophilizates were stored at -15°C for 1 h.

HPLC. HPLC analysis was performed on either one of two systems: (a) a Beckman liquid chromatograph system equipped with a model 421 gradient controller, a model 112 solvent delivery system, and a model 153 UV detector. The system used a Vydac C18 reverse-phase column (0.46 x 25 cm, 5-μm particle size; The Separations Group, Hesperia, CA) or (b) a Waters system that used a model 680 automated gradient controller, a model 6000 A and M-45 solvent delivery system, and a Lambda-Max model 480 spectrophotometer. The system was equipped with a Waters C18 μ Bondapak reverse-phase column (84176, 0.78 x 30 cm; Waters Associates, Milford, MA). Lyophilized cell extracts, representing about 10 g (wet weight) of cultured breast carcinoma cells, were reconstituted in 10 ml of 0.1% HFBA and centrifuged at 18,000 x g for 1 h at 4°C. The supernatant was loaded at a flow rate of 1.5 ml/min onto the HPLC column which was previously equilibrated with 0.1% HFBA. Five min after loading was completed, a 50-
min linear gradient from 0 to 60% acetonitrile in 0.1% HFBA was run at a flow rate of 1.5 ml/min. Fractions (1.8 ml) were collected.

Aliquots were removed from each fraction and lyophilized twice after addition of water. These were reconstituted in PBS containing 0.1% gelatin and were assayed in duplicate for GnRH immunoreactivity using the different antisera.

GnRH Radioimmunoassay. GnRH was measured by radioimmunoassay as previously described (16, 17). The antiserum used were 1076 (18), EL-14 (19) (W. E. Ellinwood, Department of Physiology, Oregon Health Sciences University, Portland, OR), and F-86 (S. S. Lynch, Department of Clinical Endocrinology, The Birmingham and Midland Hospital for Women, Birmingham, United Kingdom). Antiserum 1076 requires the middle residues Trp3 to Pro6 for effective binding (18). Antiserum EL-14 requires both the NH2 and COOH termini of GnRH (19), while antiserum F-86 recognizes the NH2 terminus of GnRH because we have found that substitution or deletion of amino acid residues 1, 2, or 3 results in a decrease in relative binding to <3% and substitutions in positions 4–10 resulted in relative binding of 34–333%.

Poly(A) RNA Isolation. RNA was extracted from tissues or from cell pellets by the single-step procedure of Chomczynski and Sacchi (20). Breast carcinoma cells, HeLa cells, JEG human placental cell line, human placenta, human hypothalamus (obtained within 24 h of death), rat hypothalamic preoptic area sections, and rat pituitary and liver tissue were used as the source of the RNA. Human hypothalamus were stored at −70°C until use. All other tissues were dissected out immediately prior to RNA extraction. Poly(A) mRNA was purified from total RNA using Hybond-MAP paper (Amer sham International) according to the method of Werner et al. (21).

S1 Nuclease Protection Assay. A 34-base oligonucleotide (Oswel DNA Service, Edinburgh, Scotland) was used as a probe in the S1 nuclease protection assay. The oligonucleotide is complementary to nucleotides 85–118 of the cloned human and rat hypothalamic mRNA and nucleotides 1127–1160 of the cloned human placental mRNA which encode amino acid residues −6 to +6 of the GnRH precursor protein (22) and has the sequence 5′-CCATAGGACCAGTGCTGGCGTGACCAGCCTTCCA-3′. The probe was 5′-end labeled to a specific activity of ~107 dpm/pmol using T4 polynucleotide kinase (Amersham International) in the presence of [γ-32P]ATP (Amersham International) and has the sequence 5′-CCATAGGACCAGTGCTGGC-

RESULTS

GnRH Immunoreactive Peptides. Several peaks of GnRH immunoreactivity eluted on HPLC separation of acidic acid extracts of ZR-75-1 and MDA-MB-231 cells (Fig. 1). In both cell lines one of these eluted with the same retention time as a synthetic GnRH standard in two HPLC systems and was detected by GnRH antisera directed at different regions of the molecule. The other immunoreactive peaks did not react with all the GnRH antisera and eluted at positions different from that of GnRH. GnRH immunoreactivity was not detected in identical blank HPLC runs conducted prior to chromatographing the cell extracts. The possibility that GnRH immunoreactivity was artifactual due to proteases in the extract which digest 125I-GnRH and thus decrease the bound radioactivity in the radioimmunoassay was excluded by demonstrating that 125I-GnRH binding by excess antiserum was unaffected by prior incubation of the radiolabeled peptide with the HPLC fractions under assay conditions.

No immunoreactivity was detectable in medium conditioned by MDA-MB-231 cells for at least 24 h (results not shown).

Fig. 1. GnRH immunoreactivity in cultured breast carcinoma cells. Approximately 10 g (wet weight) of ZR-75-1 and MDA-MB-231 cells was extracted in acidic acid and subjected to chromatography on a reverse-phase HPLC system. In this study, the extract of ZR-75-1 was chromatographed on a C18 Vydac column (a), and the MDA-MB-231 extract was chromatographed on a C18 Waters μ Bondapak column (b). Aliquots of the fractions were lyophilized and analyzed for GnRH immunoreactivity using the region-specific antisera (see "Materials and Methods"). Shaded portion of bar, region of the GnRH molecule that is detected by that antiserum. a, top, elution position of synthetic mammalian GnRH, when chromatographed on the C18 Vydac column in the presence of cell extract.
GnRH mRNA. Poly(A) mRNA extracted from MDA-MB-231 and ZR-75-1 cells and hypothalamus protected the labeled GnRH 34-mer oligonucleotide (complementary to nucleotides 85–118 of the human hypothalamic GnRH mRNA) from digestion by S1 nuclease. The oligomer was not protected by liver and pituitary mRNA (Fig. 2).

PCR amplification of first-strand cDNA from MDA-MB-231 cells, HeLa cells, JEG cells, and human placenta was conducted with two sets of oligonucleotide primers (Fig. 3B). MDA-MB-231 cells, JEG cells, and human placenta yielded expected 245- and 380-base pair products, while there was no product from HeLa cells (Fig. 3A).

Since the GnRH mRNA in human placenta is considerably larger than the product in human hypothalamus because of retention of intron I and there are a number of putative start sites in exon I (Fig. 4B), it was of interest to determine the characteristics of breast carcinoma cell mRNA.

Extension of the 34-mer oligonucleotide by AMV reverse transcriptase in the presence of MDA-MB-231 and ZR-75-1 poly(A) RNA gave a predominant ~150-base cDNA product identical to that obtained with human hypothalamic poly(A) RNA (Fig. 4A). The size of this extended cDNA is consistent with that expected from the sequence of the human hypothalamic GnRH precursor cDNA (22) in which a start site 61 bases into exon I is used and intron I excised (Fig. 4B). A faint smear in the region of 250–500 bases in length was present in the human hypothalamic and the ZR-75–1 cell line samples and suggests minor utilization of other upstream putative start sites at about 150–300 bases upstream of exon I (Fig. 4). Even larger products of between 1000 and 1300 bases were obtained with mRNA from both breast carcinoma cell lines but not with...
human hypothalamic mRNA (Fig. 4A). These correspond to mRNA isolation procedure used does not exclude nuclear RNA, the larger primer-extended cDNA could be due to the presence of immature human mRNA in the extract. However, since identical procedures were used for hypothalamic mRNA preparation and these large products were not obtained, it appears that nonexcision of the 870-base intron is a feature common to the placenta and breast cell lines but not to the hypothalamus.

**DISCUSSION**

While the presence of GnRH-binding sites in some mammary carcinoma tissues (13) provided a rationale for direct palliative effects of GnRH in certain postmenopausal patients (11, 12), a model cell biological system was required to further investigate this possibility.

We previously reported the presence of specific GnRH-binding sites in MDA-MB-231 andZR-75-1 mammary carcinoma cell lines (15). GnRH agonist was shown to decrease proliferation in MCF-7 cells which also display low-affinity GnRH-binding sites (14). We were unable to elicit inhibition of MCF-7, MDA-MB-231, andZR-75-1 with GnRH agonist (15). However, a GnRH antagonist consistently inhibited radiolabeled thymidine incorporation into MDA-MB-231 andZR-75-1 cells in the range of 10^{-5}-10^{-7} M (15). Similarly, the antagonist inhibited growth of these cells as well as MCF-7 cells at 10^{-6} M (15). The relatively high concentration of analogue necessary to demonstrate inhibition was compatible with the apparent low affinity of the GnRH-binding site (15). The apparent discrepancy between our observations of inhibition by GnRH antagonists (15) and inhibition of MCF-7 cells with GnRH agonists by another group (14) may be explicable in view of the inhibitory effects of both classes of analogues on pituitary gonadotropin secretion (10): agonists by desensitization and antagonists by competing out endogenous GnRH. Our results showing a consistent inhibition of MDA-MB-231 andZR-75-1 cells by a GnRH antagonist suggested that these breast carcinoma cells are producing GnRH or a GnRH-like peptide, the effects of which are counteracted by GnRH antagonist administration.

The current studies demonstrate that the cell extracts contain a peptide with immunological and chromatographic properties indistinguishable from those of GnRH. In two different HPLC systems, this peptide elutes at the position of synthetic mammalian GnRH and is similarly quantitated by antisera directed at the NH2 terminus of GnRH, the middle portion of GnRH and both the NH2 and COOH termini of the peptide. In contrast, other immunoreactive peaks which did not coelute with synthetic mammalian GnRH were not recognized by all the antisera. These peaks might represent unprocessed or partially processed GnRH precursor or degraded forms of GnRH. The GnRH precursor is known to be recognized best by middle-directed antisera and not by antisera which bind to the COOH terminus of the mature peptide (26).

The authenticity of the GnRH and its production by the cells was further established by the demonstration of GnRH mRNA by S1 nuclease protection assay, by PCR amplification, and by primer extension studies. The primer extension study indicates that the breast carcinoma cells predominantly transcribe short mRNA forms with intron I excised as in hypothalamic GnRH neurons but also produce longer mRNA in which intron I is retained as in the human placenta (25, 27). The primer extension studies also indicate that the main start site used by the breast carcinoma cell lines is about 60 bases into exon I as in human hypothalamus (27) but that start sites further upstream are also used as in human placenta (25, 27). The significance of the differential usage of start sites and inclusion/excision of intron I requires investigation, as does the possibility of regulation of start site usage.

The amount of GnRH peptide present in the breast carcinoma cells was very low when compared with the hypothalamus. However, mRNA levels were relatively high. This suggests that translation of the mRNA in the carcinoma cells is restrained or that the translated peptide is not adequately processed or rapidly degraded. The possibility of an inhibition of translation is suggested by our observation of high levels of mRNA transcribed from the complementary DNA strand.3

The demonstration of GnRH production by breast carcinoma cells is not without precedent since the peptide has been reported to be present in breast milk (28-30). It appears, therefore, that the GnRH gene may be expressed in breast cells during lactation and malignant transformation.

While the expression of the GnRH gene in breast carcinoma cells, which display GnRH-binding sites, points to an autocrine (31, 32) regulatory role for this peptide, confirmatory evidence for such a role requires the demonstration of GnRH secretion and the neutralization of the effects of the peptide either with specific antibodies or with GnRH antagonists. This evidence would be strengthened by documenting the effects of purified breast tumor-derived GnRH peptide on cultured breast carcinoma cells. We have shown that GnRH antagonists inhibit breast carcinoma cell proliferation (15). We could not find detectable levels of GnRH in the medium conditioned by breast carcinoma cells. This may be due to considerable dilution or rapid degradation of the peptide or may indicate that the peptide is not secreted from the cells. Recent evidence suggests that certain autocrine growth factors such as the v-sis oncogene product, a retroviral homologue of the B-chain of platelet-derived growth factor, may act on the cognate receptor at an intracellular site (33). Therefore, extracellular secretion may not be a prerequisite for their action. In view of the low concentrations of GnRH peptide in the cells we have been unable to purify sufficient material to demonstrate an effect on cell growth. In any event, if the effects are intracellularly mediated, very high levels might be required when administered extracellularly.

Cultured human breast carcinoma cells produce an array of regulatory polypeptides which have the potential of altering cellular proliferation in either an autocrine or paracrine fashion (34, 35). Thus, MCF-7 cells secrete both growth stimulatory factors such as transforming growth factor-α, insulin-like growth factor-I, and platelet-derived growth factor as well as inhibitory factors such as transforming growth factor-β (34, 35). The demonstration of GnRH gene expression in breast carcinoma cells adds another putative regulatory peptide to this series. More detailed studies are required to delineate the role of GnRH in the growth of breast carcinoma.

**ACKNOWLEDGMENTS**

We wish to thank Dr. Judy King for helpful discussions, Drs. W. Ellinwood and S. Lynch for two of the region-specific antisera, and L. Odes and M. Hamel for manuscript preparation.

3 N. Harris, C. Dutlow, K. Eidne, K-W. Dong, J. Roberts, and R. Millar, unpublished data.
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