Two Forms of Gonadotropin-releasing Hormone (GnRH) Are Expressed in Human Breast Tissue and Overexpressed in Breast Cancer: A Putative Mechanism for the Antiproliferative Effect of GnRH by Down-Regulation of Acidic Ribosomal Phosphoproteins P1 and P2

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) analogues are used for the treatment of breast, prostate, and ovarian cancer. These analogues exert their antitumor effects indirectly by inhibiting the pituitary-gonadal axis, as well as by direct inhibition of proliferation of human breast cancer cells. However, the molecular mechanisms mediating these direct antiproliferative effects are not fully understood. We found that normal and malignant human breast tissue express two forms of the neuropeptide GnRH. Quantitative reverse transcription-PCR shows that mRNA encoding the GnRH-I and GnRH-II peptides are overexpressed in cancerous versus normal tissues obtained from the same patients. To elucidate the function of these peptides in breast cancer cells, we used the atlas human cDNA expression arrays technology and studied the differentially regulated genes after GnRH treatment of MCF-7 cells. We found that a wide range of GnRH-I or GnRH-II concentrations (0.1–10 nM) inhibit the expression of mRNA encoding the 60S acidic ribosomal phosphoproteins, P1 and P2. These results were confirmed by quantitative reverse transcription-PCR, as well as Western blot analysis and immunofluorescence staining. The P1 and P2 proteins interact with elongation factors EF1 and EF2, and the level of their phosphorylation is one of the regulatory mechanisms for the overall rate of protein elongation. Thus, reduced expression of P1 and P2 proteins can affect the rate of protein translation, thereby decreasing proliferation rate of cells. Our studies may therefore suggest a putative mechanism for the direct antiproliferative effect of GnRH in breast cancer cells.

INTRODUCTION

GnRH-1 (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), also named luteinizing hormone-releasing hormone, was originally isolated from the mammalian hypothalamus and plays a pivotal role as the physiological regulator of reproduction (1, 2). This peptide is synthesized and released by hypothalamic neurosecretory cells and reaches the pituitary gland by way of a specialized portal system to induce the synthesis and secretion of the gonadotropic hormones, which regulate gonadal functions (1–3). In addition to its expression in the hypothalamus, GnRH-I was also found to be expressed in extra-hypothalamic regions of the central nervous system (4), as well as in non-neuronal tissues, such as placenta (5), ovary (6), mammary gland (7), and lymphoid cells (8). In addition, GnRH-I and its receptor were found to be expressed in a number of malignant tissues and cell lines, including cancers of the breast, ovary, endometrium, and prostate. The function of GnRH-I in extra-pituitary tissues is unclear, although in recent years, an autocrine/paracrine function has been suggested (for review, see Refs. 9 and 10). GnRH analogues are used for the therapy of sex hormone-dependent tumors of the breast and prostate. These analogues exert their antitumor effects indirectly by inhibiting the production of sex hormones after desensitization of the pituitary gland by their continuous administration. In addition, direct inhibitory effects of native GnRH-I and its agonistic analogues on the proliferation of human breast cancer cell lines were first demonstrated by Miller et al. (11). Since then, the evidence for the inhibition of proliferation of a variety of human breast cancer cell lines by agonistic and/or antagonistic analogues of GnRH have been substantiated (for review, see Refs. 9 and 10). The molecular mechanisms mediating the direct antiproliferative effects of GnRH-I analogues in human cancers are currently considered to involve its interference with the mitogenic signal transduction pathways (12, 13).

Recently, a second form of GnRH (GnRH-II; His5, Trp7, and Tyr7-GnRH-I), was identified in the brain of mammalian species (14–16). The GnRH-II gene was cloned from human (17) and monkey (18) brains. Originally, GnRH-II was isolated as a second form of GnRH from the chicken brain (19). It has since been found in all vertebrate classes from primitive fish to humans (20). The wide distribution of this neuropeptide overall vertebrate classes demonstrates its conservation over the years of evolution and may imply that its physiological functions are most important.

The goal of this study was to determine whether human breast tissue and cell lines express the two forms of GnRH and to elucidate possible functions of these neuropeptides in breast cancer cells. By using the atlas human cDNA expression arrays technology, we studied the differentially regulated genes after GnRH treatment. Our results demonstrate, using a variety of techniques, that normal and malignant human breast tissues, as well as breast cell lines, express both GnRH-I and GnRH-II. Treatment of breast carcinoma cells (MCF-7) by either of the two forms of GnRH resulted in a strong down-regulation of the acidic ribosomal phosphoproteins P1 and P2. The 60S subunit of the eukaryotic ribosomes contains three acidic ribosomal proteins, P0, P1, and P2, that exist in phosphorylated states (21). The P1 and P2 proteins, which play an important role in the elongation step of protein synthesis, interact with eucaryotic elongation factors EF1 and EF2 and are required for aminoacyl-tRNA binding and EF2-dependent GTPase activity, as well as for polypeptide synthesis (22, 23). Our results may therefore propose a putative mechanism for the antiproliferative effect of GnRH.

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma cell lines MCF-7 and MDA-MB-231, and the nontumorigenic epithelial cell line MCF-10A, were used. The MCF-7 and MDA-MB-231 cell lines were maintained in DMEM (Sigma Chemical Co., St. Louis, MO), supplemented with 10% FCS (Biological
Industries, Beth Haemek, Israel), whereas the MCF-10A cell line was maintained in a 1:1 mixture of Ham’s F-12 (Sigma Chemical Co.) and DMEM with 2.5 mM L-glutamine and supplemented with 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone. All media were supplemented with penicillin (100 I/U/ml) and Streptomycin (100 µg/ml; Life Technologies, Inc., Paisley, Scotland). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2/95% air.

Analysis of Gene Expression Using the Human Atlas cDNA Expression Array. Poly(A)+RNA was extracted from the human MCF-7 cell line that were maintained in phenol red free DMEM in the presence of 10% charcoal-stripped FCS, before and after treatment with 10 nM GnRH-II for 24 h. We used the Atlas Pure Total RNA Labeling System (Clontech Laboratories, Inc., Palo Alto, CA) according to manufacturer’s recommendations. After DNAse treatment, cDNA was prepared from poly(A)+RNA preparations that were prepared from either untreated or GnRH-II-treated MCF-7 cells. Hybridizations to the Atlas Human cDNA Expression Arrays membranes (Human 1.2 Array III, Cat # 7855–1, Clontech Laboratories, Inc.) were performed according to the user manual, and the expression pattern was visualized by autoradiography. Normalization of results, with reference to the housekeeping genes, was performed by using the AtlasImage Software (Clontech Laboratories).

RNA Preparation. Total RNA was extracted (24) from the human breast cell lines MCF-7, MDA-MB-231, and MCF-10A and from normal and cancer breast tissues that were obtained during routine biopsies and from plastic surgeries at the Rabin Medical Center. We have collected six different cases in which malignant and normal tissues could be separated from the same patient. Four additional samples were obtained from plastic surgeries. The use of human tissue in these studies was reviewed, and the protocol was approved by the Rabin Medical Center Human Investigation Committee (Helsinki protocol #2474). To verify the results obtained from the human atlas cDNA expression array membranes, we extracted RNA, using the Atlas Pure Total RNA Labeling System (Clontech Laboratories, Inc.) from MCF-7 cells maintained in DMEM lacking phenol red in the presence of 10% charcoal-stripped FCS. The cells were treated with either GnRH-I or GnRH-II (0.01, 0.1, 1, 10, and 100 nM) for 24 h. Control cells were left untreated. MCF-7 cells were also treated with 100 nM of the GnRH-I antagonist, Cetorelix (SB75; ASTA Medica AG, Frankfurt, Germany).

Quantitative RT-PCR and Southern Analysis. GnRH-I and GnRH-II experiments: We used quantitative RT-PCR to amplify the levels of endogenous GnRH-II and GnRH-I mRNA that may be present in the human breast tissue samples and cell lines and compare the expression levels of the two GnRH forms in normal versus malignant breast tissues. The expression of the ribosomal protein, S-14 (25), served as an internal control. Each reaction contained four oligonucleotide primers, two for each of the GnRH forms (GnRH-I or GnRH-II) and two for the S-14 internal control. The PCR conditions were: cDNA equivalent to 0.5 µg of RNA was amplified by PCR for 32–35 cycles; the annealing temperatures were 62°C and 60°C for GnRH-II and GnRH-I reactions, respectively. The final MgCl2 concentration was 2.5 mM, and the Taq DNA polymerase used in this study was the BIO-X-ACT DNApolymerase (Bioline UK Ltd., London, United Kingdom). The Southern hybridization procedure, performed for P1 and P2 proteins, was similar to that described for the GnRH forms. Hybridizations were performed at 55°C for P1 probe and at 60°C for the P2 probe.

Oligonucleotide Primers. The specific human GnRH-I, GnRH-II, and S-14 oligonucleotide primers that were used in the PCR reactions were described earlier (24). The predicted size of the GnRH-I band is 248 bp, that of GnRH-II is 197 bp, and that of S-14 is 143 bp. Sense and antisense primers were selected to be located on different exons of GnRH-I, GnRH-II, and S14 to avoid false positive results caused by DNA contamination (Fig. 1A). The other oligonucleotides primers were: P0–5’ TTGTTGTCACAGAGGAGG 3’ and 5’ GTAGCCTATCTGCACGAG 3’ corresponding to nucleotides 340–357 (sense) and 752–770 (antisense), respectively (21); the predicted size of the band is 431 bp P1–5’ CAAAGTGTTCGCGTTCCTTC 3’ and 5’ GAACATGTATAAAAAGGAGG 3’ corresponding to nucleotides 19–36 (sense) and 476–494 (antisense), respectively (21); the predicted size of the band is 476 bp.

PCRT = 0, P1, P2, and L37 proteins: To verify the results obtained from the human atlas cDNA expression array membranes, we performed quantitative RT-PCR for the ribosomal proteins using the S14 gene as an internal control. The amount of RNA (1–500 ng) and number of PCR cycles (15–38 cycles) were calibrated for the quantitative RT-PCR assay of the ribosomal proteins P0, P1, P2, and L37 transcripts obtained from MCF-7 cells. The ethidium bromide bands were quantified, and the average relative signals were correlated with the amount of total RNA and with the number of PCR cycles as demonstrated by the graphs in Fig. 5, D and E. The experimental PCR conditions were: cDNA equivalent to 10–50 ng of RNA was amplified by PCR for 32 cycles for P1 and P2 and for 28 and 25 cycles for P0 and L37, respectively; the annealing temperatures were 54°C, 55°C, 62°C, and 52°C for P0, P1, P2, and L37 reactions, respectively. The final MgCl2 concentration was 2.5 mM, and the Taq DNA polymerase used in this study was the BIO-X-ACT DNApolymerase (Bioline UK Ltd., London, United Kingdom). The Southern hybridization procedure, performed for P1 and P2 proteins, was similar to that described for the GnRH forms. Hybridizations were performed at 55°C for P1 probe and at 60°C for the P2 probe.

Fig. 1. Expression of GnRH-I and GnRH-II genes in human breast tissue. A, schematic representation of the GnRH-I, GnRH-II, and S14 transcripts. GnRH-I, GnRH-II, and S14 cDNA are shown with introns (lines), exons (squares), poly(A) tail (wavy line), and location of the PCR fragments (shaded [squl]). The length in bp of the introns, exons, and each of the PCR fragments is indicated. B, Southern blot hybridization of amplified GnRH-I, GnRH-II, and GnRH-I and S14 cDNA fragments. Amplified GnRH-I, GnRH-II, and S14 cDNA fragments from human normal breast (Lanes 3 and 4), breast cancer (Lanes 5 and 6), and normal breast tissue surrounding cancer tissue (Lanes 1 and 2) were hybridized to a human GnRH-I (top panel), GnRH-II (middle panel), and S14 (bottom panel) [24] total RNA of MCF-7, MCF-7, and MCF-7 cells. The ethidium bromide bands were quantified, and the average relative signals were correlated with the amount of total RNA and with the number of PCR cycles as demonstrated by the graphs in Fig. 5, D and E. The experimental PCR conditions were: cDNA equivalent to 10–50 ng of RNA was amplified by PCR for 32 cycles for P1 and P2 and for 28 and 25 cycles for P0 and L37, respectively; the annealing temperatures were 54°C, 55°C, 62°C, and 52°C for P0, P1, P2, and L37 reactions, respectively. The final MgCl2 concentration was 2.5 mM, and the Taq DNA polymerase used in this study was the BIO-X-ACT DNApolymerase (Bioline UK Ltd., London, United Kingdom). The Southern hybridization procedure, performed for P1 and P2 proteins, was similar to that described for the GnRH forms. Hybridizations were performed at 55°C for P1 probe and at 60°C for the P2 probe.
P2-5’ TCCGCCGAGCCGCGC 3’ and 5’ TGACGGGAGCAGAATT 3’ corresponding to nucleotides 54–70 (sense) and 422–439 (antisense), respectively (21); the predicted size of the band is 386 bp L37–5’ CAGAACGAGATGCGCA 3’ and 5’ CAGACATTTATTCGAGC 3’ corresponding to nucleotides 19–38 (sense) and 338–358 (antisense), respectively (Kato, S. GenBank, direct submission); the predicted size of the band is 340 bp. The oligonucleotide probes for hybridization were: GnRH-I-5’ CCAAGTCTAGAATAAGGCCC 3’ corresponding to nucleotides 2091–2111, GnRH-II-5’ CAGAGGCTCCTGGCTGAGGCTGCTGCTG 3’ corresponding to nucleotides 2965–2985, P1–5’ ATCTTTACTTCCCTGCTAATCGTC 3’ corresponding to nucleotides 192–212, and P2–5’ CAGGGCCAGAACCAGCAGCA 3’ corresponding to nucleotides 320–339.

DNA Sequencing. The appropriate cDNA fragments of GnRH-I and GnRH-II from normal human breast tissue were extracted from the gels using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and subcloned into PGEM-T vector by using the PGEM-T Easy Vector System I (Promega Corp.). Nucleotide sequencing of the specific PCR bands was performed by automated direct DNA sequencing, according to the manufacturer’s recommendations (model 377; PE Applied Biosystems and Perkin-Elmer Corp., Foster City, CA).

Cell Processing for GnRH Determination. Twenty confluent culture dishes (100 mm) of MCF-7 cells were immersed in ice-cold 0.1 N HCl and homogenized by a Polytron homogenizer. After centrifugation (12,000 × g, 30 min at 4°C), the supernatant was pumped onto columns of Sep-Pak C-18 cartridges (Waters Corp., Milford, MA) and later processed through RP HPLC as described earlier (15, 24). All fractions were evaporated to a volume of 0.1 ml and reconstituted with 0.1 ml PB (pH 7.4) containing 0.1% of bovine γ-globulin, and the concentration of GnRH-I and GnRH-II was determined by RIA using the appropriate antiserum. The elution positions of the synthetic peptides were determined later by application of 1 μg of GnRH-I and of GnRH-II.

Radioiodination and RIA. Iodination of synthetic GnRH-I or GnRH-II was carried out using the chloramine-T method. Free iodine was removed on a Sep-Pak C-18 cartridge (Waters Corp.), and the 125I-labeled peptides were separated from the unlabeled peptides by HPLC (15). GnRH concentrations in samples of MCF-7 cell extracts were determined by RIA as described previously (26).

Antibodies. The following antisera were used throughout this study: A polyclonal antibody against GnRH-I, prepared and characterized in our laboratory, was used for RIA (26). GnRH-II or salmon GnRH did not displace any of the bound 125I-GnRH-I, even at a concentration that exceeded by 1,000 times the GnRH-I concentration that is needed for displacing 50% of the tracer (20 ng versus 20 pg). A monoclonal antibody against GnRH-I, kindly provided by Dr. H. F. Urbansky, was used at dilutions ranging from 1:4000 to 1:10,000 for the immunofluorescence studies; the specificity of this antibody (HU/4H) was reported elsewhere (27). Two polyclonal antibodies against GnRH-II were used. One antibody, aCl6, was kindly provided by Dr. K. Okuawara, and its specificity was defined previously (15, 28). Additional specificity tests in our laboratory demonstrated that GnRH-I did not displace any of the bound 125I-GnRH-II, even at a concentration that exceeded 2,500 times the GnRH-II concentration that is needed to displace 50% of the tracer (20 ng versus 8 pg). The second antisera, KLII-2, was prepared and characterized in our laboratory (29). Specificity tests of this antibody have demonstrated that GnRH-I did not displace any of the bound 125I-GnRH-II, even at a concentration that exceeded 1,000 times the GnRH-II concentration that is needed to displace 50% of the tracer (30 ng versus 30 pg). Salmon GnRH cross-reacted with this antiserum by 0.003% and with antibody aCl6 by 0.013%. We have used dilutions ranging from 1:4000 to 1:10,000 of the GnRH-II antibodies for the immunohistochemical studies. Monoclonal antibodies against acidic phosphoproteins were kindly provided by Dr. T. Uchimi. Whereas anti-P recognizes the P0, P1, and P2 proteins, anti-P1 recognizes the P1 protein only (30). We have used dilutions ranging from 1:1,000 to 1:2,000 of the anti-P antibody for the immunoblot studies and 1:500 to 1:1,000 of the anti-P1 antibody for the immunofluorescence studies.

Confocal Double Fluorescence Immunocytochemical Analysis. MCF-7 cells were analyzed by double fluorescence immunocytochemistry using confocal microscopy. The cells were plated on round glass coverslips (13 mm) coated with poly-L-lysine (15 μg/ml) in 24-well culture plates. Two days later, the cells were fixed by the addition of 4% paraformaldehyde in 0.1 M PB (pH 7.4; 30 min), washed (5 min ×3) with PB, and permeabilized for 3 min with 0.5% Triton X-100. After washing (×3), the cells were incubated for 2 h at room temperature in a blocking medium (PBS containing 10% normal goat serum, 2% BSA, 1% glycine, and 0.5% Triton X-100) to saturate nonspecific binding sites for IgG. The primary antibodies were added for 12–15 h at 4°C, and the cells were washed (5 min ×3) with 0.1 M PB. The cells were then incubated for 2 h at room temperature with fluorescein- or rhodamine-conjugated secondary antibody as follows: goat antimouse conjugated to Cy3 (red fluorescence; Jackson ImmunoResearch Laboratories, West Grove, PA), goat antirabbit conjugated to Oregon Green (green fluorescence; Molecular Probes, Eugene, OR), or with both antibodies. The cells were washed with PB and coverslipped with fluorescence-mounting medium. They were visualized under a confocal laser microscopy (Zeiss 510; Zeiss, Oberkochen, Germany) equipped with filters for fluorescein and Cy3 epifluorescence. Image analysis was performed using the standard system operating software provided with the Zeiss 510 microscope (version 2.01). To determine the specificity of the signals, we included control groups in which the antibodies were preabsorbed with excess (2–100 μg) of GnRH-I or GnRH-II for 24 h. Additional control sections were incubated without the first antibody, with normal mouse/rabbit serum, or with an irrelevant antibody.

Immunohistochemical Procedure. For the immunohistochemical studies, we have used 14 different samples of breast cancer and 4 samples of breast tissue taken during plastic surgeries. The tissues were fixed in 10% of buffered formalin, embedded in paraffin, and cut into 4-μm sections that were reacted with specific antibodies for GnRH-I and GnRH-II. The immunohistochemical procedure was performed using the DAKO CSA System (Cat. #K1500; DAKO, Glostrup, Denmark) according to the manufacturer’s recommendations. The DAKO CSA System is a sensitive immunocytochemical visualization system based on peroxidase-catalyzed deposition of biotin, which in turn is reacted with peroxidase-conjugated streptavidin, resulting in a greatly amplified signal. Because the DAKO CSA System is intended for primary antibodies of mouse origin, we adapted it torabbit primary antibodies by using the CSA Rabbit Link (Cat. #K1498; DAKO). To determine the specificity of the signals, we preabsorbed aliquots of the antibody with excess (10–100 μg) of GnRH-I or GnRH-II for 24 h. Additional control sections were incubated without the first antibody or with an irrelevant antibody. The sections were also stained with hematoxylin to demonstrate the morphological structure of the tissues.

Immunoblotting Analysis. MCF-7 cells in 100-mm plates were grown in phenol red free DMEM containing 10% charcoal-treated FCS. The cells were treated with either GnRH-I or GnRH-II (10 nm) for 48 h. Control cells were left untreated. The cells were washed with cold PBS and lysed in 800 μl of lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 200 μM NaVO3, and 100 mM NaF]. After an SDS-PAGE (18% SDS-polyacrylamide gel) and transfer to nitrocellulose, the samples were immunoblotted with the anti-P antibody.

Statistical Analysis. All of the data are presented as the mean ± SD and were analyzed by t test or one-way ANOVA using the Excel version 6.0 software package (Microsoft Corp., Seattle, WA). P < 0.05 was considered statistically significant.

RESULTS

mRNA for GnRH-I and GnRH-II in Human Breast Tissues. Total RNA preparations derived from normal and cancerous breast tissues obtained from routine biopsies and from plastic surgeries were reverse transcribed to generate cDNA. The cDNA products were used as templates to the PCR by using specific primers for GnRH-I, GnRH-II, and for the ribosomal protein S14 that served as an internal control. Southern hybridizations were performed sequentially on the same membrane using 32P-labeled oligonucleotide probes, specific to S14, GnRH-I, or GnRH-II. Results are shown in Fig. 1. The RT-PCR and Southern hybridizations demonstrate that GnRH-I (Fig. 1B, top panel) and GnRH-II (Fig. 1B, middle panel) are expressed in the human normal breast (Fig. 1B, Lanes 3 and 4), breast cancer (Fig. 1B, Lanes 5 and 6), and in normal breast tissue surrounding cancer tissue.
The ribosomal protein S14 that served as an internal control was expressed, as expected, in all cDNA preparations (Fig. 1B, bottom panel) and the ribosomal protein S14 cDNA fragments from human breast cell lines: MCF-7 (Lanes 1 and 2), MCF-10A (Lanes 3 and 4), and MDA-MB-231 (Lanes 5 and 6) were hybridized to a human GnRH-I (top panel), GnRH-II (middle panel), and S14 (bottom panel) $^{32}$P-labeled oligonucleotide probes. The predicted size of GnRH-I, GnRH-II, and S14 fragments is 248, 197, and 143 bp, respectively. Lanes 1, 3, and 5 contain PCR products with GnRH-I and S14 primers, whereas Lanes 2, 4, and 6 contain PCR products with GnRH-II and S14 primers; Lane 7 contains PCR products without the RT enzyme, and Lane 8 contains PCR products without cDNA. The last two served as negative controls.

Coexpression of GnRH-I and GnRH-II by Human Breast Cell Lines. Total RNA was extracted from human breast carcinoma cell lines MCF-7 and MDA-MB-231 and from the nontumorigenic epithelial cell line MCF-10A. The ribosomal protein S14 that served as an internal control was expressed as expected, in all cDNA preparations (Fig. 1B, bottom panel). As can be observed in Fig. 1B, the level of GnRH-I and GnRH-II in breast cancer tissues is higher than in tissues that were obtained from normal breast. In addition, the level
of GnRH-I and GnRH-II in normal breast tissue obtained by plastic surgery seems to be lower than that in normal breast surrounding cancer. However, the levels of GnRH forms expressed by the nontumorous cell line MCF-10A are found to be similar to the amount produced by the tumorous cell lines MCF-7 and MDA-MB-231 (Fig. 2A). This discrepancy may be explained by the heterogeneity and complexity of the breast cancer tissue compared with the less complex breast carcinoma cell line.

Acid extracts of 20 confluent tissue culture dishes (100 mm) of MCF-7 cells were eluted through RP-C18 columns on HPLC using an isocratic elution program that is known to separate synthetic GnRH-I from GnRH-II. The concentrations of GnRH-I or GnRH-II in the eluate of MCF-7 cell extracts were determined by using RIA systems that are specific for GnRH-I or GnRH-II. Fig. 2B demonstrates that the elution profiles of the immunoreactive neuropeptides that were extracted from MCF-7 cells were identical to those of the synthetic peptides (open and solid arrows, for GnRH-I and GnRH-II, respectively). These results demonstrate the existence of the two neuropeptides, GnRH-I and GnRH-II, in MCF-7 cells, at a concentration of ~2.5 pg/10^6 cells.

Further support for the translation of the mRNA of the two neuropeptides comes from confocal double fluorescence immunocytochemical analysis for GnRH-I and GnRH-II in MCF-7 cells. Fig. 2C demonstrates GnRH-II (Fig. 2C, a and d) and GnRH-I (Fig. 2C, b and e) immunoreactivity in MCF-7 cells. GnRH-I monoclonal antibody was reacted with goat-antimouse-Cy3 (red fluorescence), whereas GnRH-II polyclonal antibody was reacted with goat-antirabbit Oregon Green (green fluorescence). Panels c and f in Fig. 2C demonstrates the superimposed pictures of GnRH-I and GnRH-II staining. We could not observe heterogeneity among cells within the MCF-7 cell line. The cells were immunoreactive for both GnRH forms, but different levels of intensity could be observed within the MCF-7 cells. MCF-7 cells that were reacted with either PBS, normal mouse/rabbit sera, or with an irrelevant antibody followed by secondary antibodies did not result in any staining. In addition, preabsorption of GnRH-I antibody with excess of synthetic GnRH-I abolished the immunoreactive staining (data not shown). A similar procedure that was carried out with the antibody to GnRH-II has also exhibited the expected specificity of the antibody.

mRNAs Encoding the GnRH-I and GnRH-II Peptides Are Overexpressed in Cancer As Compared with Normal Tissue Obtained from the Same Patient. Immunohistochemical studies that were carried out on paraffin sections obtained from normal and breast cancer tissues using antibodies specific for GnRH-II (Fig. 3, A, C, and E) and GnRH-I (Fig. 3, B, D, and F) revealed a strong immunoreactive staining in the breast cancer tissue (Fig. 3, C–F) and a modest staining in the normal tissue (Fig. 3, A and B). The sections were stained also by hematoxylin to demonstrate nuclei of cells. The GnRH-I and GnRH-II immunoreactivity is observed in the epithelial cells of the breast tissue. Diverse intensity of staining could be observed in different cases of breast cancer that were examined, as can be observed in Fig. 3, C and D, and compared with Fig. 3, E and F, representing two different cancer specimen. Immunoreactivity for GnRH-I and GnRH-II was observed in 8 of 14 of the breast samples that were examined using immunohistochemistry. On the other hand, 6 of 6 breast cancer samples were found to express mRNA for both GnRH forms. The discrepancies between these two observations are probably because of the different sensitivity of the two methodologies. In addition, it is important to note that we could not find any specimen that expressed only one of the two forms of GnRH.

In six different specimens obtained during routine biopsies, it was possible to separate the malignant tissue from the normal tissue of the same patient. Total RNA was extracted, and quantitative RT-PCR was used to compare the expression level of the two forms of GnRH in normal versus malignant tissues. To calibrate the experimental conditions for the quantitative RT-PCR assay and use the assay in its linear phase of amplification, we performed PCR, using cDNA equivalent to 250 ng of total RNA, for increasing numbers of amplification cycles (Fig. 4A). The average relative signal of GnRH-I, GnRH-II, and S14 correlates with the number of PCR cycles and is demonstrated by a graph (Fig. 4A). For the quantitative assay, the cDNA was amplified by PCR for 32 cycles using specific oligonucleotide primers for GnRH-I, GnRH-II, and S14. PCR and Southern blot hybridization of amplified GnRH-I (Fig. 4B, top panels), GnRH-II (Fig. 4B, middle panels), and the ribosomal protein S14 (Fig. 4B, bottom panels) were performed. The normalized values (corrected according to the S14 intensity) are presented as fold of increase compared with the normal tissue.

Differentially Regulated Genes in MCF-7 Cells Treated with GnRH: Both GnRH-II and GnRH-I Strongly Reduce the Expression Level of the Acidic Ribosomal Phosphoproteins P1 and P2. To explore the possible effects of GnRH-II on gene expression of MCF-7 cells, we exposed MCF-7 cells to GnRH-II (10 nM) for 24 h.
Poly(A)+ RNA was prepared from GnRH-II-treated and, from the control, untreated cells and reverse transcribed to 32P-labeled cDNA. We have used a human atlas cDNA expression array, i.e., a positively charged nylon membrane spotted with 1200 different cDNAs and the reverse transcribed products were hybridized to the atlas membranes. The differential pattern of expression between untreated cells and GnRH-II-treated cells was visualized by autoradiography (Fig. 5, A and B). The normalized results (corrected against the housekeeping genes, spotted on the control panels, Fig. 5C) revealed that among the genes that were affected, GnRH-II induced the down-regulation of mRNA encoding the 60S ribosomal proteins P1, P2, and L37 in GnRH-II-treated cells (panel B) as compared with untreated cells (panel A, visualized in coordinate 6m, 6n, and 5m, respectively). Fig. 5C presents the control hybridizations for the untreated cells (top panel) and GnRH-II treated cells (bottom panel). To confirm the results of the human atlas cDNA expression array, we have determined the proper conditions for the quantitative RT-PCR assays (Fig. 5D). Because the family of the acidic ribosomal phosphoproteins includes, in addition to P1 and P2, also the P0 protein, we have added the P0 protein to the genes that were further investigated.

After calibration of the experimental conditions (Fig. 5D), we measured the levels of the ribosomal proteins P0, P1, P2, and L37 mRNA in MCF-7 cells, after treatment with increasing doses (0.01–100 nm) of either GnRH-I or GnRH-II for 24 h. The PCR results were visualized by etidium bromide staining. It is clearly evident that the P0 (Fig. 6A) and L37 (data not shown) genes remained unaffected after incubation with increasing doses of either GnRH-I or GnRH-II. In contrast, GnRH-I caused a clear down-regulation of P1 and P2 (Fig. 6, B and C, respectively). Southern hybridization was performed for the down-regulated proteins P1, P2, and for SI4 using specific 32P-labeled probes (Fig. 6, B and C, middle panels). The normalized values (relative to the control SI4 expression) are presented as bar graphs (Fig. 6, bottom panels). Similar effects on the expression of P1 and P2 proteins were also observed after the incubation of the cells with GnRH-II (data not shown). These results demonstrate that the acidic ribosomal phosphoproteins P1 and P2 mRNA levels in cells, treated with either GnRH forms, were significantly down-regulated in comparison with untreated cells. To examine whether the effects of both GnRH forms on P1 and P2 expression are receptor mediated, we measured the P1 and P2 mRNA level in MCF-7 cells after treatment with the GnRH-I antagonist Cetrorelix (SB-75). The MCF-7 cells were treated with 100 nm Cetrorelix for 24 h with or without either of the two GnRH forms. The mRNA levels of P1 and P2 in MCF-7 cells remained unaffected when the cells were treated with 100 nm Cetrorelix (Fig. 7, Lanes 5 and 6). These results may indicate that the GnRH-II effects are also mediated via the GnRH-I receptor, unless the Cetrorelix can also antagonize the yet unknown, specific human GnRH-II receptor. Recently, the cloning of the primate GnRH-II receptor was reported (31, 32) and revealed a ubiquitous tissue distribution, including the breast tissue. However, the human GnRH-II receptor remains still unknown.

The down-regulation of P1 and P2 proteins by both GnRH forms was further investigated at the protein level by Western blot and immunofluorescence staining. Protein extracts obtained from MCF-7 cells that were treated with either 10 nm GnRH-I or GnRH-II for 48 h, were analyzed by Western blot, using an anti-P antibody that recognizes the three acidic ribosomal phosphoproteins P0, P1, and P2 (Fig. 8A). Control cells were left untreated. Treatment with either GnRH-I or GnRH-II resulted in a significant decrease in the expression levels of the P1 and P2 proteins, but not of the P0 protein as can be observed in the Western blot (Fig. 8A), and in the graph that summarizes three independent experiments (Fig. 8B). Additional support for the down-regulation effect of GnRH-I and GnRH-II on the P1 protein expression came from immunofluorescence staining of MCF-7 cells, untreated or treated with GnRH-II for 48 h. Using the anti-P antibody, there is a strong reduction in the perinuclear staining of the P1 protein after GnRH-II treatment (Fig. 8, C and D). Similar results were obtained after GnRH-I treatment (data not shown).

Fig. 5. Analysis of gene expression using the atlas human cDNA expression array. A, untreated cells; B, GnRH-II-treated cells. Down-regulation of the 60S ribosomal proteins P1, P2, and L37 in GnRH-II-treated cells (B) compared with untreated cells (A) can be seen at coordinates 6m, 6n, and 5m, respectively. C, the control hybridizations for untreated cells (top panel) and the GnRH-II-treated cells (bottom panel). The housekeeping genes are located in boxes 1–3, 6–8, and 11–13 and stand for Ubiquitin, phospholipase A2, hypoxanthine-guanine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, brain-specific tubulin α 1 subunit, HLA class I histocompatibility antigen C-4 α subunit, cytoplasmic β-actin, M, 23,000 highly basic protein, and 40S ribosomal protein S9, respectively. The negative controls are: λ DNA and pUC18 DNA, located in boxes 5 and 10, respectively. Human genomic DNA is spotted in boxes 4 and 9. D and E, the calibration of RNA amounts and PCR cycles for the quantitative RT-PCR assay of the ribosomal proteins P0, P1, P2, and L37 transcripts obtained from MCF-7 cells. D demonstrates P0, P1, P2, and L37 products using increasing amounts of cDNA (1–500 ng of total RNA). E, PCR was performed for increasing numbers of amplification cycles using cDNA that is equivalent to 50 ng of total RNA. The ethidium bromide bands were quantified by AlphaEase program (Alpha Innotech, San Leandro, CA). The average relative signal of P0, P1, P2, and L37, correlated with the amount of total RNA and the number of PCR cycles, is demonstrated by graphs.
DISCUSSION

We have demonstrated, using RT-PCR and Southern hybridization, that GnRH-I and GnRH-II are expressed in normal and malignant human breast tissues. In addition, GnRH-I and GnRH-II are coexpressed in the human breast carcinoma cell lines MCF-7 and MDA-MB-231 and by the nontumorigenic epithelial cell line MCF-10A. The nucleotide sequences of the GnRH fragments were found to be identical to the known GnRH-I and GnRH-II sequences. The presence of the two neuropeptides, GnRH-I and GnRH-II, in the MCF-7 cells was further demonstrated by HPLC, followed by double immunofluorescence staining.

GnRH-I immunoreactivity was previously demonstrated in samples of human breast cancer (33), as well as in human breast cancer cell lines (34). GnRH-I mRNA was found in the human breast cancer cell lines MDA-MB-231 and ZR-75–1 (35). In addition, it was shown previously by our group that GnRH-I is present in the milk of human, cow, and rat (36) and that the GnRH-I gene is expressed in the mammary gland of pregnant and lactating rats (37). Human breast cell lines that express the two forms of GnRH provide an excellent model system to evaluate the mechanisms mediating the specific differential expression of human GnRH-II and GnRH-I, as well as the role of various neurotransmitters, growth factors, hormones, and other biological substances that may regulate directly or indirectly the synthesis or secretion of the two GnRH forms. After screening of seven human neuronal cell lines, we have demonstrated recently two cell lines, TE-671 medulloblastoma and LAN-1 neuroblastoma cells, that...
coexpress mRNA encoding the two GnRH forms, GnRH-I and GnRH-II (29). Subsequently, the differential regulation of the GnRH-I and GnRH-II genes by cyclic AMP at the promoter, mRNA, and peptide levels were studied using the TE-671 cells as a model system (24). Using similar methodologies, the MCF-7, MDA-MB-231, and MCF-10A cell lines can be used for transfection with GnRH-I or GnRH-II promoter constructs to investigate the differential regulation of their genes after stimulation with various biological substances.

Here, we studied the presence of GnRH-I and GnRH-II in human breast tissue. In the six samples that were examined, the GnRH-I and GnRH-II mRNA levels were significantly higher (2- to 7-fold) in malignant breast tissue as compared with normal breast tissue that was obtained from the same patient. These results may be the consequence of the overall high protein expression and enhanced transcription machinery that exist in cancer cells. All of the six samples of breast cancer that were examined were found to express mRNA for both GnRH forms, and we did not detect any specimen that expresses only one form of GnRH. On the other hand, immunoreactive GnRH-I and GnRH-II were observed in 8 of 14 of the breast cancer samples that were examined by immunohistochemistry. The discrepancies between these observations are probably because of the different sensitivity of the two methodologies. In all positive cases, GnRH-I and GnRH-II immunoreactivity were more intense in the malignant as compared with the normal tissues.

Direct antiproliferative effects of GnRH-I agonist and antagonists on a variety of human breast cancer cells have been shown by many investigators (9–11, 38–43). The main molecular mechanisms mediating the direct antiproliferative effects of GnRH-I analogues in human cancers are considered to be their interference with the mitogenic signal transduction pathways. GnRH-I analogues were found to reduce the growth factor-induced tyrosine kinase activity in breast cancer cell lines (12, 13). Growth factor-induced tyrosine phosphorylation is counteracted by GnRH analogues through activation of a phosphotyrosine phosphatase, which is coupled to the GnRH-I receptor through a Gi protein in human reproductive tumors (44). GnRH-I analogues were found to reduce the expression of growth factor receptors and their mRNA in ovarian (45) and prostatic (46) cancer cell lines. The activation of mitogen-activated protein kinase by EGF was blocked in ovarian and endometrial cancer cells treated with GnRH agonist (47). The EGF-induced expression of the immediate early gene c-fos was inhibited in human cancer cells treated with GnRH-I agonist or antagonist. (46, 48). Recently, GnRH-II has been also shown to possess antiproliferative effects in normal and neoplastic ovarian epithelial cells (49).

To elucidate additional possible autocrine/paracrine functions of the two forms of GnRH, we used the atlas human cDNA expression arrays technology to study the genes that are differentially regulated after GnRH treatment. The results obtained from the gene array experiments were confirmed by quantitative RT-PCR, Western blot, and immunofluorescence staining. The results reveal a strong down-regulation of the expression of the acidic ribosomal phosphoproteins P1 and P2 caused by both GnRH-I and GnRH-II. The mRNA and protein levels of the third member of this family, the P0 protein, remained unchanged after GnRH treatments.

The large ribosomal subunits of higher eukaryotes have three acidic proteins that exist in phosphorylated states (P proteins). The properties and amino acid sequence of the three acidic proteins, P1, P2, and P0, have been characterized and were found to have highly homologous sequences at the COOH-terminal region (21). The P1 and P2 proteins interact with eukaryotic elongation factors EF1 and EF2 and are required for aminoacyl-tRNA binding and EF2-dependent GTPase activity, as well as for polypeptide synthesis (22, 23). Cross-linking of 60S ribosomal subunits showed that P1 and P2 are present as homodimers, each of which interacted with monomeric P0 (50). Thus, the P1 and P2 proteins participate in interactions with elongation factors and are a major component of the eukaryotic ribosomal translation domain. Provided that the level of phosphorylation of these proteins is one of the regulatory mechanisms of the overall rate of protein elongation, it is conceivable that changes in the rate of production of these proteins could disrupt the normal control of the protein synthesis. Enhanced/reduced expression of the ribosomal proteins could indicate a higher/lower rate of overall translation, which may be related to the proliferation rate of the cells under investigation. The inhibition of lymphosarcoma cell proliferation, induced by glucocorticoid, was demonstrated to be related to the repression of rRNA transcription and translation of mRNA-encoding ribosomal protein, including mRNA coding for the P2 protein (51–53).

Additional studies are required to elucidate the pathways mediating the down-regulation effect of the two forms of GnRH on P1 and P2 proteins. Whereas it seems that GnRH induces its antiproliferative effects directly by down-regulating the ribosomal phosphoproteins in breast cells, it is also possible that this effect is actually mediated indirectly by a reduction of GnRH receptor density in breast cells that may be caused by the prolonged stimulation of GnRH. We propose a putative autocrine/paracrine role for GnRH in breast cells proliferation. The GnRH peptides influence the expression level of the acidic ribosomal phosphoproteins P1 and P2 and, thus, interfere with the elongation process of proteins and disrupt protein synthesis. This cascade of events, controlled by GnRH, may provide a putative mechanism for the regulation of the proliferation rate of breast cancer cells.

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