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Autocrine Role of Gonadotropin-Releasing Hormone and Its Receptor in Ovarian Cancer Cell Growth

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We have recently proposed an autocrine role of gonadotropin-releasing hormone (GnRH) and its receptor (GnRH-R) in human ovarian surface epithelium. In the present study, we examine the presence and role of a GnRH/GnRH-R loop in epithelial ovarian cancer cells, OVCAR-3. A dose-dependent biphasic response in GnRH and GnRH-R mRNA levels were observed after treating with GnRH agonist [GnRHa, (D-Ala^6)-GnRH], for 24 h. High concentrations of GnRHa (10^{-9} *M* and 10^{-7}) decreased the GnRH and GnRH-R mRNA levels, whereas a low concentration (10^{-11} *M*) resulted in an upregulation of GnRH and GnRH-R genes expression. Cotretment with the competitive antagonist, antide, prevented the biphasic effect induced by GnRHa, confirming the specificity of the response. In addition, GnRHa treatment resulted in a time- and dose-dependent inhibition on OVCAR-3 cells growth. A significant inhibition of proliferation was detected as early as the d 2 of treatment. Treatment with 10^{-7} *M* GnRHa induced DNA fragmentation in OVCAR-3 cells, suggesting that the GnRHa-induced antiproliferation in OVCAR-3 cells was mediated by apoptosis. Again, this effect was prevented by cotreatment of antide. Taken together, our findings strongly support the notion that GnRH acts as an autocrine/paracrine regulator of ovarian cancer cell proliferation.

Key Words: Gonadotropin-releasing hormones, GnRH receptor; ovarian cancer; growth inhibition; apoptosis.

Introduction

In addition to its well-documented role in the regulation of gonadotropin synthesis and secretion *(1)*, gonadotropin-releasing hormone (GnRH) has been suggested to have a functional role in normal and malignant reproductive tissues. This concept is based on the detection of GnRH gene transcripts, synthesis of the GnRH, and the multitude of effects attributed to GnRH receptor (GnRH-R)-mediated signaling in extrapituitary tissues *(2–6)*. In the ovary, GnRH modulates both basal and gonadotropin-stimulated steroidogenesis *(7,8)* and induces transcription of several genes involved in the follicular maturational process and ovulation *(9,10)*. GnRH and synthetic analogs have been shown to exert an antiproliferative effect in sex steroid-sensitive tumors *(11,12)*. The proposed antitumor mechanism of GnRH is thought to be a desensitization or downregulation of the GnRH-R in the pituitary, resulting in a decline in the gonadotropin secretion and gonadal steroid production that serve as tumor growth factors. However, this may not be the only antigrowth mechanism of GnRH and its analogs. In postmenopausal women, in whom gonadal steroids are lacking, GnRH exerted a growth inhibitory effect on breast tumor cells *(13)*. GnRH also has been shown to inhibit growth of a number of GnRH-R-bearing tumor cell lines including ovarian cancer in vitro, further substantiating the direct effect of GnRH by a mechanism independent of suppression of gonadal hormones *(14–16)*. The exact mechanism of the antiproliferative action of GnRH, however, still remains to be elucidated.

The presence of an autocrine/paracrine regulatory system based on GnRH in normal and malignant reproductive tissues suggests that GnRH and its receptor may be regulated in these extrapituitary tissues similarly to those in the hypothalamus and pituitary. In the hypothalamus, numerous hormones have been shown to regulate the synthesis and release of GnRH either directly, involving GnRH neurons, or indirectly, involving neurons that communicate with the GnRH neurons *(17)*. In addition, GnRH has been shown to regulate its own synthesis in a biphasic manner in immortalized hypothalamic GT1-7 neurons *(18)*. It is well documented that the number of GnRH-R is both up- and downregulated by the homologous ligand, GnRH, in the pituitary of many mammalian species *(1,19)*. In the pituitary, stimulation with physiologic concentrations of GnRH in vitro is followed by a biphasic pattern of changes in receptor numbers. Initially, a downregulation of receptors is associated with a desensitization of the gonadotropes to GnRH, followed by upregulation *(20,21)*. In normal and malignant reproductive tissues, however, the regulation of GnRH and its receptor is poorly understood.

We have recently proposed that GnRH and its receptor may have an autocrine role in human ovarian surface epi-

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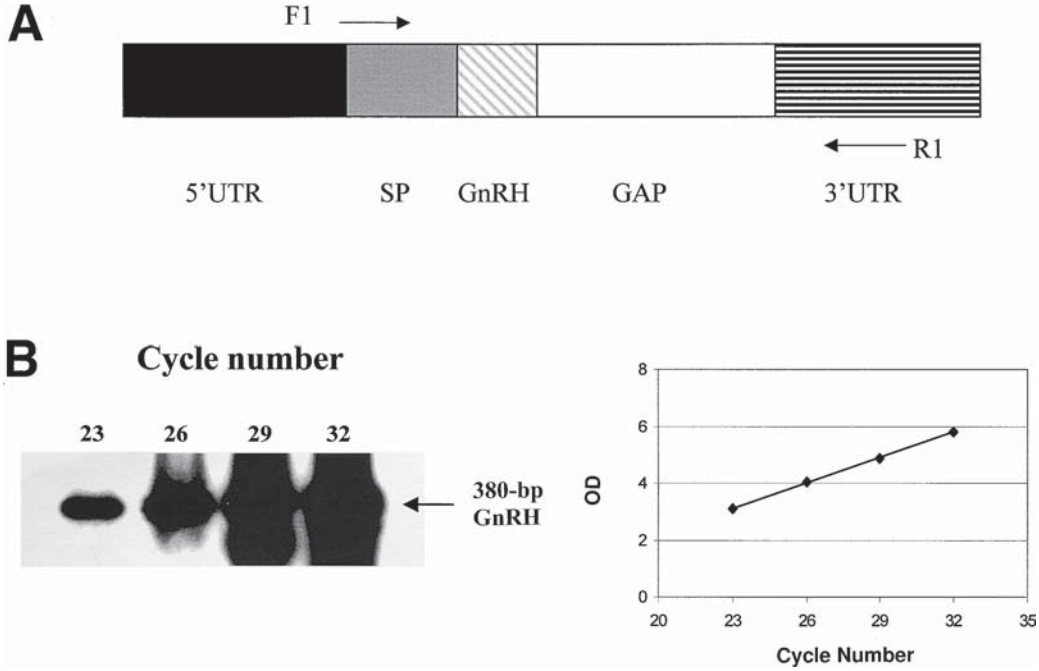

Fig. 1. Validation of semiquantitative reverse transcriptase (RT)-PCR for GnRH. Total RNA was isolated, reverse transcribed, and aliquots were amplified using different numbers of cycles as described in Materials and Methods. **(A)** Locations of the primers employed (F1 and R1) are given. **(B)** A linear relationship was observed between PCR products and amplification cycles when plotted.

thelium (OSE) *(22)*. In the present study, we explore the possibility of the presence of a GnRH/GnRH-R loop in epithelial ovarian tumors that originate from normal OSE. As in the hypothalamic-pituitary axis and normal OSE, we first investigated homologous regulation of GnRH and its receptor in the human ovarian cancer cell line. Furthermore, to investigate the physiologic significance, we studied growth regulatory effects of GnRH and its possible antigrowth mechanism. Understanding of the regulation of GnRH and its receptor, and of the growth inhibitory mechanisms of GnRH on ovarian cancer cells, may contribute to better hormonal treatment protocols.

Results

Validation of Polymerase Chain Reaction

To determine the conditions under which polymerase chain reaction (PCR) amplification for GnRH and β-actin mRNA were in the logarithmic phase, 2.5 μg of total RNA was reverse transcribed, and aliquots (2 μL) were amplified using different numbers of cycles. A linear relationship between PCR products and amplification cycles was observed in both GnRH (Fig. 1) and β-actin mRNA (data not shown). Twenty-six cycles for GnRH and 18 cycles for β-actin were employed for quantification. The standard curve for GnRH-R was constructed by a coamplification of a fixed amount of competitive cDNA (mutant GnRH-R) with the addition of serial dilutions of the target cDNA (native GnRH-R), as described previously *(22)*. To titrate the amount of the competitor, a fixed amount of first-strand cDNA from OVCAR-3 cells (2 μL from 2.5 μg of RNA) was coamplified with serial dilutions of competitive cDNA. Increasing the amount of the mutant cDNA resulted in decreased amplification of the native GnRH-R from the sample cDNA. A similar degree of amplification was observed when 0.006 pg of mutant cDNA was added, and this concentration was employed for competitive PCR for GnRH-R transcript (Fig. 2).

Homologous Regulation of GnRH and GnRH-R mRNA

Treatment with the GnRH agonist induced a biphasic regulation pattern for GnRH and GnRH-R mRNA levels. High concentrations of (D-Ala^6)-GnRH (10^{-7} and 10^{-9} *M*) decreased GnRH and GnRH-R mRNA levels, whereas a low concentration (10^{-11} *M*) resulted in an upregulation of GnRH and its receptor (Fig. 3A,B). To confirm the specificity of the biphasic effect by the GnRH agonist, the cells were treated with different concentrations of the GnRH agonist together with GnRH antagonist, antide. Cotreatment with antide abolished the biphasic response in the OVCAR-3 cell line (Fig. 3B). Antide alone had no effect on GnRH-R mRNA levels (Fig. 3B).

Cell Proliferation Assays

As shown in Fig. 4, (D-Ala^6)-GnRH inhibited the growth of OVCAR-3 cells in a dose-dependent manner. A significant inhibition of proliferation was detected as early as the d 2 of treatment at concentrations of 10^{-7} and 10^{-9} *M*. At lower concentrations (10^{-11} *M*), reduction of cell growth at d 2 was insignificant. The antiproliferative effect of

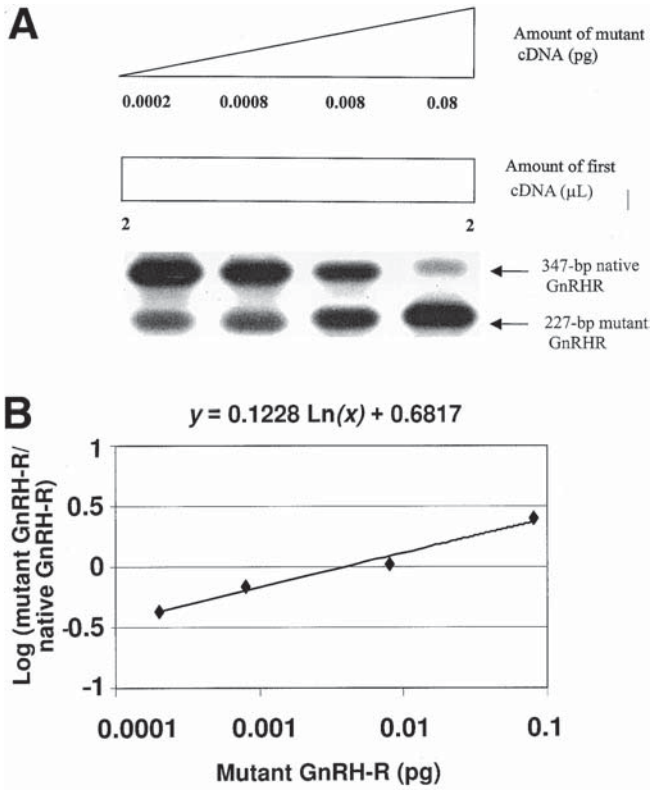

Fig. 2. Validation of competitive RT-PCR for GnRH-R transcript. To titrate the amount of the competitor, a fixed amount of first-strand cDNA from OVCAR-3 cells (2 μL from 2.5 μg of RNA) was coamplified with serial dilutions of competitive cDNA. **(A)** Increasing the amount of the mutant cDNA resulted in decreased amplification of the native GnRH-R from the sample cDNA. **(B)** A similar degree of amplification was observed when 0.006 pg of mutant cDNA was added.

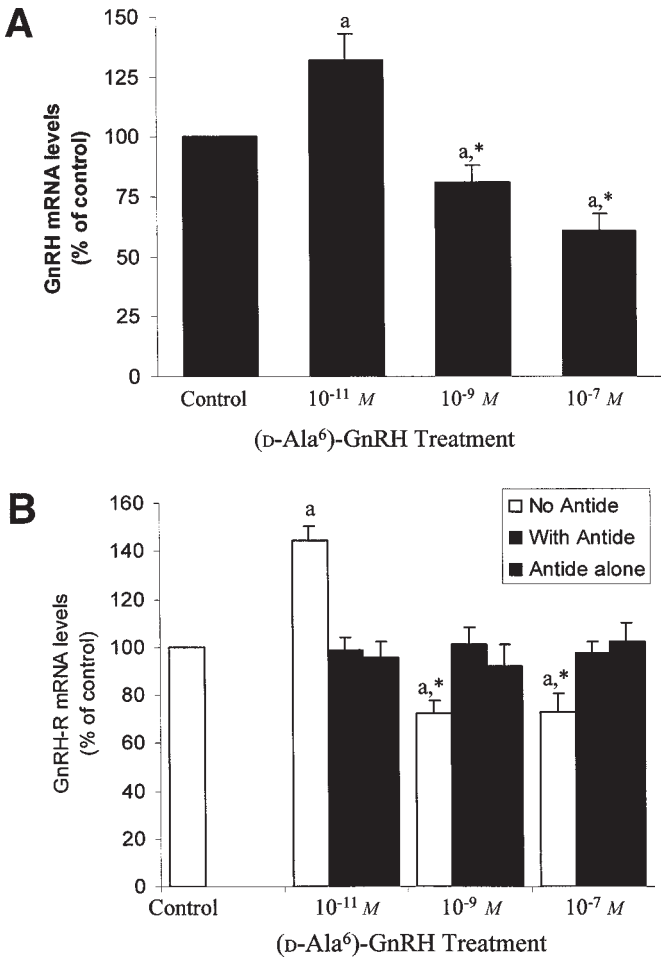

Fig. 3. Effect of GnRH on GnRH mRNA **(A)** and GnRH-R mRNA **(B)** in OVCAR-3 cells. OVCAR-3 cells were cultured and treated with different concentrations of the (D-Ala^6)-GnRH (10^{-11}, 10^{-9} or 10^{-7}, *M*) for 24 h (A). To confirm specificity of the GnRH agonist, OVCAR-3 cells were incubated with medium containing (D-Ala^6)-GnRH (10^{-11} *M*) + antide (10^{-9} *M*), (D-Ala^6)-GnRH (10^{-9} *M*) + antide (10^{-7} *M*), and (D-Ala^6)-GnRH (10^{-7} *M*) + antide (10^{-7} *M*) for 24 h. Cell cultures were also treated with antide alone. Control cultures were treated with vehicle (distilled water). Data are shown as the mean of three individual experiments with duplicate samples and are presented as the mean ± SD. $^a p < 0.05$ vs control; $^* p < 0.05$ vs 10^{-11} *M* (D-Ala^6)-GnRH.

(D-Ala^6)-GnRH was also time dependent as the growth inhibitory effect was increased with time of treatment. Inhibition of growth continued through to d 4 and was further evident at d 6 of treatment. On d 6, 10^{-7} *M* (D-Ala^6)-GnRH reduced growth to 62% of control.

Downregulation of GnRH-R mRNA

To determine whether the inhibition of OVCAR-3 cell growth was associated with altered GnRH-R gene expression, OVCAR-3 cells were treated with 10^{-7} *M* (D-Ala^6)-GnRH in a time-dependent manner. As shown in Fig. 5, treating the cells with (D-Ala^6)-GnRH resulted in a time-dependent decrease in GnRH-R mRNA levels. Densitometric analysis of the transcript revealed that GnRH-R mRNA levels were decreased to 62% of control after 1 d of treatment. The GnRH-R mRNA levels were further downregulated to 34% of control after 6 d of treatment.

DNA Fragmentation Assays

To analyze whether the growth inhibitory effects of the (D-Ala^6)-GnRH were associated with programmed cell death, cells were treated with the GnRH agonist for 6 d and genomic DNA was isolated, fractionated by agarose gel electrophoresis. The cells on poly-HEMA-treated culture dishes served as positive controls. As shown in Fig. 6, DNA fragmentation was observed in OVCAR-3 cells treated with the GnRH agonist. To confirm whether the cell death was a receptor-mediated event, cells were treated with the GnRH agonist plus antide. No DNA fragmentation was observed under this condition (Fig. 6).

Discussion

In addition to its role in pituitary gonadotropin synthesis and secretion, GnRH is thought to be an autocrine/paracrine modulator of ovarian function *(7)*. In the present study, we

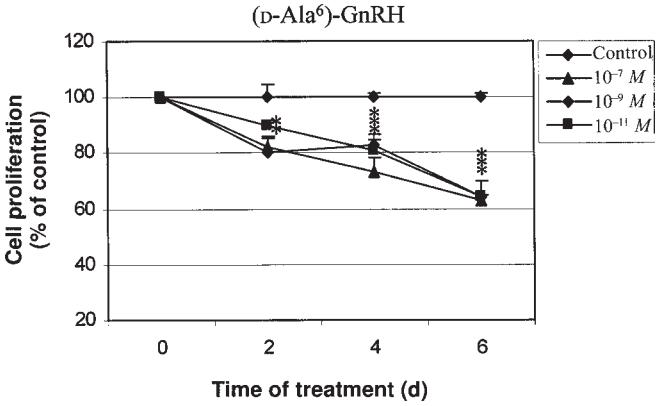

Fig. 4. Effect of the (D-Ala^6)-GnRH on growth of OVCAR-3 cells. Cells were treated with different concentrations of the GnRH agonist (D-Ala^6)-GnRH for 2, 4, and 6 d on a daily basis. Control cultures were treated with vehicle (distilled water). The (D-Ala^6)-GnRH inhibited the growth of OVCAR-3 cells in a time- and dose-dependent manner. Significant reduction in growth vs control was found at d 2 for the (D-Ala^6)-GnRH at concentrations of 10^{-7} and 10^{-9} *M* and at d 4 and 6 for all concentrations. $^{*}p < 0.05$ vs control.

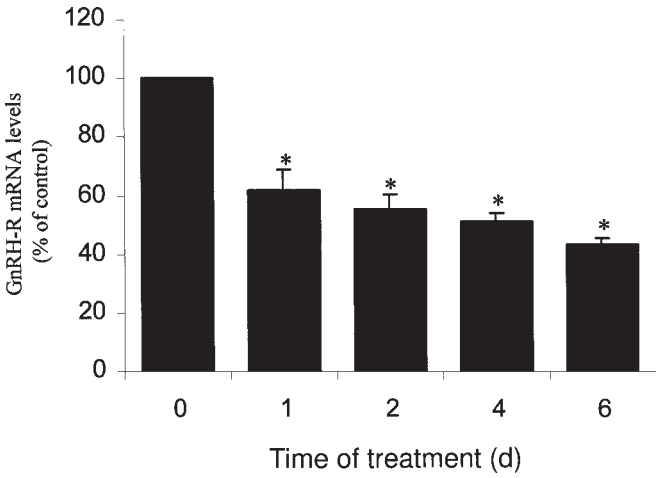

Fig. 5. Effect of continuous treatment of the (D-Ala^6)-GnRH on GnRH-R mRNA levels. Cells were treated with (D-Ala^6)-GnRH (10^{-7} *M*) in a time-dependent manner. Densitometric analysis of the transcript revealed that GnRH-R mRNA levels were decreased to 62% of control after 1 d of treatment. By d 6, GnRH-R mRNA levels were further downregulated to 34% of control after 6 d of treatment. $^{*}p < 0.05$ vs control.

demonstrate, for the first time, that as in hypothalamic-pituitary axis and normal OSE, GnRH and GnRH-R mRNA are regulated by their homologous ligand in a biphasic manner in the ovarian epithelial cancer cell line, OVCAR-3. In addition, we show that the GnRH agonist has a direct antiproliferative effect on ovarian cancer cell growth by inducing apoptosis.

One of the interesting findings of the present study is the demonstration that GnRH gene expression is regulated by GnRH itself in the ovarian cancer cell line, as in the hypothalamus. In the rat hypothalamus, GnRH regulates its own synthesis and release through an ultrashort loop feedback mechanism *(23–25)*. In addition, GnRH has been shown to have biphasic effects on GnRH secretion from immortalized hypothalamic neurons in a time- and dose-dependent manner *(18)*. Likewise, it is well documented that GnRH-binding sites are both up- and downregulated by GnRH in the pituitary of various species *(1,19)*. In general, low doses or pulsatile treatment of GnRH upregulate its receptor, whereas high doses or continuous treatment downregulate the receptor numbers. Changes in the GnRH-R mRNA level have been explained as at least part of the mechanisms underlying up- and downregulation of GnRH-R numbers. Pulsatile treatment with 10 nM GnRH induced an increase in GnRH-R mRNA levels in rat pituitary cells *(26)*, whereas continuous treatment with the same concentration of GnRH for 48 h decreased levels of the receptor mRNA in cultured sheep pituitary cells *(27)*. A biphasic regulation pattern of GnRH-R mRNA levels has also been demonstrated in human ovarian cells *(6)*. In the present study, a low dose of the GnRH agonist (10 p*M*) increased GnRH-R mRNA levels. By contrast, higher doses (1 and 100 n*M*) of the GnRH agonist induced a statistically significant decrease in GnRH-R mRNA levels in OVCAR-3 cells (Fig. 3B). This regulation appears to be receptor mediated because cotreatment with a competitive GnRH antagonist, antide

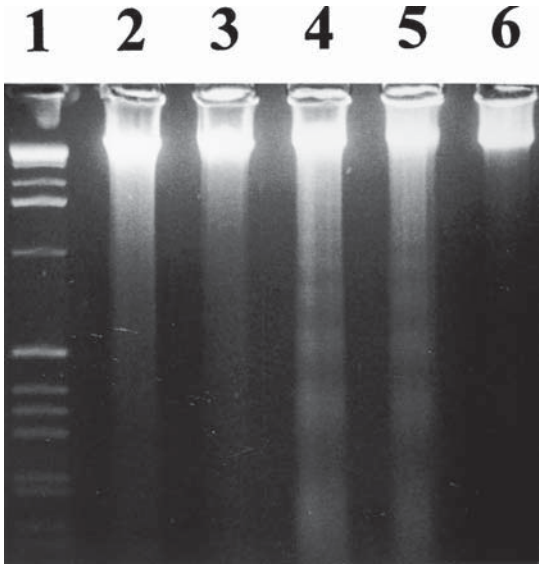

Fig. 6. Induction of DNA fragmentation in OVCAR-3 by the GnRH analogs. Cells were treated with the GnRH analogs for 6 d on a daily basis, and genomic DNA was isolated, fractionated by agarose gel electrophoresis. Lane 1, DNA molecular weight marker; lane 2, negative control; lane 3, antide treatment (10^{-7} *M*); lane 4, positive control from the cell on poly-HEMA-treated culture dishes; lane 5, (D-Ala^6)-GnRH treatment (10^{-7}*M*); lane 6, treatment with (D-Ala^6)-GnRH (10^{-7}*M*) plus antide (10^{-7}*M*). DNA fragmentation was observed in cells treated with the GnRH agonist but not antide. Cotreatment with antide abolished the effect of the GnRH agonist.

(28), blocked the biphasic effect of GnRH. Our results suggest that similar to the hypothalamic and pituitary GnRH-R, alterations in the GnRH-R mRNA levels are involved in the regulation of responsiveness to GnRH in normal ovarian *(6,22)* and ovarian epithelial cancer cells. Taken together, our studies strongly suggest the presence of an autocrine/paracrine regulatory system based on GnRH in the human ovary.

Recently, it has been suggested that GnRH in extrapituitary regulates cell growth and proliferation through its receptor. In fact, GnRH analogs have been shown to be effective in treating GnRH-R-bearing tumors including carcinomas of the ovary, breast and endometrium *(13–16)*. It has been demonstrated that in vivo, a GnRH agonist has an inhibitory effect on the growth of OVCAR-3 cells in the nude mouse *(29)*. Using in vitro cell proliferation assays in this study, we demonstrated that GnRH agonist inhibits growth of the ovarian epithelial cancer cell line, OVCAR-3, in a time-dependent manner (Fig. 4). This antiproliferative effect of GnRH agonists appear to be associated with altered GnRH-R mRNA levels in that continuous treatment with GnRH agonist induced a time-dependent downregulation of the GnRH-R mRNA (Fig. 5). The parallelism in the growth inhibition of OVCAR-3 cells and downregulation of receptor mRNA suggests a direct correlation between these two parameters. Our results are consistent with the pituitary aT3-1 cell, in which inhibition of the cell growth correlated with the downregulation of GnRH-R mRNA *(30)*. An agonist-induced decrease in receptor number could occur at the posttranslational, translational, or gene transcriptional level. Downregulation of GnRH-R mRNA levels in OVCAR-3 cells by the GnRH agonist in the present study suggests that loss of receptors, at least in part, can be attributed to decreased receptor mRNA expression. Taken together, our results suggest that inhibition of OVCAR-3 cell growth by continuous treatment of the GnRH agonist is accompanied by downregulation of GnRH-R mRNA levels.

The exact mechanism of the growth inhibitory effect of GnRH analogs, however, is still not clear. It has been suggested that as in the pituitary, where downregulation of receptors inhibits gonadotropin secretion, downregulation and/or activation of the receptor in cancer cells may mediate direct antiproliferative effects. Signaling by means of GnRH could activate a downstream phosphotyrosine phosphatase in GnRH-R-bearing tumors, thereby counteracting the effects of growth factors that are mediated through tyrosine kinase *(31–34)*. In addition, recent data have demonstrated the involvement of the tyrosine kinase/phosphatase system in signal transduction in the activation of the Fas ligand/Fas gene, which triggers apoptosis in a variety of cell types *(35)*. This suggests that the cellular response to GnRH-R and Fas may be mediated through the same intracellular signal transduction pathway in terms of inducing apoptosis. In fact, GnRH analogs have been shown to induce apoptotic cell death in a number of gynecologic tumors that express GnRH-R *(36,37)*.

To elucidate the mechanism of the growth inhibitory effect of GnRH agonist, DNA fragmentation was investigated by agarose gel electrophoresis in cells continuously treated with (D-Ala^6)-GnRH. As shown in Fig. 6, at a high concentration (10^{-7} *M*) (D-Ala^6)-GnRH but not antide induced DNA fragmentation, which is a hallmark of apoptosis. This effect was abolished by cotreatment with competitive antagonist, antide, suggesting that the action of GnRH agonist is mediated through receptor activation and/or downregulation. Whether homologous downregulation of GnRH-R directly mediates this effect remains to be elucidated. Considering that GnRH analogs induce the expression of immunoactive Fas ligand in Fas/GnRH-R-bearing tumors *(38,39)*, it is likely that the GnRH analog exerts its antiproliferative action by inducing apoptosis mediated through the Fas ligand–Fas system in ovarian cancer cells. Future experiments on this mechanism are warranted to evaluate the presence of the Fas ligand–Fas system in these cells.

In summary, GnRH and its receptor mRNA were regulated in a biphasic pattern by GnRH in a dose-related manner. The GnRH agonist had a direct inhibitory effect on the growth of the ovarian cells in a time- and dose-dependent manner, and this effect was associated with altered GnRH-R mRNA levels. Furthermore, we demonstrated that the growth inhibitory effects of the GnRH agonist were associated with programmed cell death. Our findings strongly suggest that GnRH can act as an autocrine/paracrine regulator of ovarian cancer cell growth. The elucidation of the role of this system and the means by which GnRH analogs inhibit the growth of ovarian cancer cells may contribute to better treatment protocols for ovarian cancer.

Materials and Methods

Cell Culture and Treatments

The human ovarian epithelial carcinoma cell line, OVCAR-3, was cultured at 37°C in medium 199 supplemented with 5% fetal bovine serum (FBS), 100 U/mL of penicillin G, and 100 μg/mL of streptomycin (Life Technologies, Burlington, Canada) in a humidified atmosphere of 5% CO_2-95% air. The OVCAR-3 cell line was chosen for this study because this cell line has been shown to express GnRH and GnRH-R *(40)*. To study the homologous regulation of GnRH and its receptor mRNA by a GnRH agonist, 2×10^5 OVCAR-3 cells were plated onto 35-mm culture dishes. After a preincubation of 48 h, the cells were treated with (D-Ala^6)-GnRH (Sigma-Aldrich, Oakville, Canada) at concentrations of 10^{-7}, 10^{-9}, and 10^{-11} *M* for 24 h. To confirm the specificity of the GnRH agonist, the cells were simultaneously treated with different concentrations of (D-Ala^6)-GnRH (10^{-11}, 10^{-9}, or 10^{-7} *M*) plus the GnRH antagonist (antide; 10^{-9} or 10^{-7} *M*) (Sigma-

Aldrich) for 24 h. The preliminary studies were performed to determine the optimal concentration of antide to block the effect of (D-Ala^6)-GnRH. To determine whether the inhibition of OVCAR-3 cell growth was associated with altered GnRH-R gene expression, OVCAR-3 cells were incubated in medium containing 10^{-7} *M* (D-Ala^6)-GnRH for 1–6 d on a daily basis. Control cultures were treated with vehicle (distilled water). The experiments were repeated three times, each with duplicate samples.

RT-PCR Amplification and Quantification

Total RNA was prepared from cultured cells using an RNaid kit (Bio/Can, Mississauga, Canada) according to the manufacturer's suggested protocol. Briefly, cells were disrupted in lysis buffer (4 *M* guanidine thiocynate, 25 m*M* sodium citrate [pH 7.0], 0.5% *N*-lauroyl sarcosine, and 0.1 *M* β-mercaptoethanol), followed by acid-phenol extraction. RNA was eluted from the top aqueous phase on RNA matrix and dissolved with ribonuclease-free water. The RNA concentration was measured based on absorbance at 260 nm, and its integrity was confirmed by agarose-formaldehyde gel electrophoresis. Total RNA (2.5 μg) was reverse transcribed into first-strand cDNA (Pharmacia Biotech, Morgan, Canada) following the manufacturer's procedure. Total RNA (2.5 mg in 8 μL) was heated at 65°C for 10 min and centrifuged for 1 min at 10,000*g*. Dithiothreitol (1 μL), oligo-dT (1:25 dilution, 1 μL), and bulk mixture (5 μL) were added and incubated for 1 h at 37°C. After incubation, the tube was boiled for 10 min to inactivate reverse transcriptase and stored at –20°C until further use.

To compare the GnRH mRNA levels, one set of primers was designed based on the published sequence of human hypothalamic GnRH *(41)*, and quantitative RT-PCR was performed. Primers for GnRH were as follows:

1. Sense: 5'-ATTCTACTGACTTGGTGCGTG-3' (F1).
2. Antisense: 5'-GGAATATGTGCAACTTGGTGT-3' (R1).

Primers for β-actin were designed based on published sequences *(42)*, as previously described *(43)*. PCR for β-actin was performed to rule out the possibility of RNA degradation and was used to control the variation in mRNA concentration in the RT reaction, as previously described *(43)*. The cDNA was amplified in a 50-μL PCR reaction containing 2.5 U of *Taq* polymerase (Life Technologies) and its buffer (1.5 m*M* $MgCl_2$, 2 m*M* deoxy-NTP, and 50 pmol of specific primers). PCR amplification for GnRH was carried out with denaturing for 1 min at 94°C, annealing for 35 s at 53°C, extension for 90 s at 72°C, and a final extension for 15 min at 72°C for 26 cycles. For GnRH-R, competitive RT-PCR was performed.

The standard curve for GnRH-R was constructed by a coamplification of a fixed amount of competitive cDNA (mutant GnRH-R) with the addition of serial dilutions of the target cDNA (native GnRH-R), as described previously *(22)*. Primers for GnRH-R were as follows:

1. Sense: 5'-GTATGCTGGAGAGTTACTCTGCA-3' (P44F).
2. Antisense: 5'-GGATGATGAAGAGGCAGCTGAAG-3' (P45R).

cDNA (2 μL) was coamplified with 0.006 pg of the competitor (mutant GnRH-R) cDNA. The PCRs were carried out in a 50-μL PCR reaction containing 2.5 U of *Taq* polymerase and its buffer (1.5 m*M* $MgCl_2$, 2 m*M* deoxy-NTP, and 50 pmol of specific primers) with denaturing for 1 min at 94ºC, annealing for 35 s at 60°C, extension for 90 s at 72°C, and a final extension for 15 min at 72°C for 33 cycles. Amplified PCR products were subjected to Southern blot analysis. Ten microliters of PCR products was fractionated on a 1.5% agarose gel and stained with ethidium bromide. The PCR products were transferred to a nylon membrane and hybridized with digoxigenin-labeled cDNA probe for human GnRH, GnRHR, and β-actin *(6,43,44)* following the manufacturer's recommended procedure (Roche Molecular Biochemicals, Laval, Canada). After washing, the membranes were exposed to Kodak Omat X-ray film (Eastman Kodak, Rochester, NY). PCR products were quantified using a computerized visual light densitometer (model 620, Bio-Rad, Richmond, CA).

Cell Proliferation Assays

The growth of OVCAR-3 cells was determined by measuring the DNA content as described previously *(45)*. Briefly, OVCAR-3 cells were plated in 24-well plates at 3×10^4 cells/well in 0.5 mL of medium 199 supplemented with 5% FBS, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. After a 24-h preincubation period, the cells were serum starved for 16 h and treated with different concentrations of the GnRH agonist, (D-Ala^6)-GnRH (10^{-7} to 10^{-11} *M*) for 2, 4, and 6 d on a daily basis. Control cultures were treated with vehicle (distilled water). On the day of collection, the cells were washed with phosphate-buffered saline (PBS) three times and trypsinized with 0.5 mL of TRTPCK trypsin (2.5 mg/mL), Cooper Biomedical) for 15 min at room temperature. The cell lysates were diluted with 3 mL of PBS, and 150 μL (20 μg/mL) of Hoechst 33258 (Sigma-Aldrich) was added. Amounts of DNA were measured using a DNA spectrophotofluorometer (American Instrument, MD) at an excitation wavelength of 354 nm and an emission wavelength of 458 nm. Each experiment was repeated four times with triplicate samples.

DNA Fragmentation Assays

OVCAR-3 cells were plated in 60-mm culture dishes at 2×10^5 cells in 2 mL of medium 199 supplemented with 5% FBS, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. After a 24-h preincubation period, the cells were treated with the GnRH analogs for 6 d on a daily basis. At the end of d 5 of the assay, the cells in the positive control plates were transferred to poly-HEMA-treated culture dishes (Sigma-Aldrich) to trigger apoptosis *(46)*. Genomic DNA was isolated at the end of d 6. Briefly, cells were lysed

with digestion buffer (100 m*M* NaCl, 10 m*M* Tris-Cl [pH 8.0], 25 m*M* EDTA [pH 8.0], 0.5% sodium dodecyl sulfate, 0.1 μg/mL of proteinase K) overnight and incubated at 55°C. The lysate was extracted with phenol/chloroform, precipitated with ethanol, and resuspended with TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) buffer. Samples were then RNase A (100 μg/mL) treated for 1 h at 37°C, phenol chloroform extracted, ethanol precipitated, and dissolved in TE (500 ng/μL). Equal amounts of each genomic DNA were fractionated on a 1.5% agarose gel and stained with ethidium bromide.

Data Analysis

GnRH mRNA levels were expressed as the ratio of GnRH to β-actin. The amount of GnRH-R transcript was calculated from the ratio of the target to competitive cDNA. Expression levels of GnRH and GnRH-R mRNA were expressed as the percentage change from the control value. Data are shown as the mean of three individual experiments, each with duplicate samples, and are presented as the mean ± SD. In the proliferation study, values are expressed as the percentage of growth compared with the control value and are the mean ± SD of four individual experiments, each with triplicate samples. The data were analyzed by analysis of variance followed by Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant.

Acknowledgments

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