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Advances in Brief

Inhibition of Growth and Proliferation of EcRG293 Cell Line Expressing High-Affinity Gonadotropin-releasing Hormone (GnRH) Receptor Under the Control of an Inducible Promoter by GnRH Agonist (D-Lys^6)GnRH and Antagonist (Antide)[1]

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Abstract

The mechanism by which gonadotropin-releasing hormone (GnRH) agonists and antagonists inhibit tumor cell growth and proliferation is controversial. Direct mediation of the antitumor effects through the high-affinity GnRH receptors has been questioned because of the low level of expression of receptors on the tumor cells. We have developed a human kidney embryonic cell line (EcRG293) that expresses high-affinity GnRH receptor under the control of an inducible promoter activated by muristerone A. Treatment of this cell line with either GnRH agonist (D-Lys^6)GnRH or GnRH antagonist (Antide) resulted in a significant, time-dependent decrease in cell proliferation in muristerone A-induced cells but not in the uninduced cells, which do not express the GnRH receptor. These data suggest strongly that the antitumor effect of GnRH agonists and antagonist is specific, direct, and mediated through high-affinity GnRH receptors present on the cell membranes of tumor cells.

Introduction

GnRH[3] is a hypothalamic decapeptide. It is well established that this hormone plays a central role in the regulation of reproduction by stimulating the synthesis and secretion of pituitary gonadotropins, luteinizing hormone, and follicle-stimulating hormone. This action of GnRH is mediated by its binding to high-affinity membrane receptors belonging to the G-protein-coupled seven-transmembrane receptor family. The gonadotropins, in turn, regulate the gametogenic and endocrine functions of the gonads. GnRH receptors have been reported to be expressed on extrapituitary tissues including the testes, ovary, brain, adrenal, and placenta in addition to the anterior pituitary (1, 2). However, the physiological relevance of the existence of GnRH receptors in extrapituitary tissues remains unresolved.

Evidence that the use of GnRH agonists and antagonists may be effective in the treatment of endometriosis and hormone-dependent cancers has been forthcoming in recent years (3). It was hypothesized originally that the major mechanism of action of GnRH agonists in the inhibition of tumor growth was to desensitize the pituitary with a consequent decline in gonadotropin secretion/gonadal hormone production. An alternative direct mechanism is suggested by the results of radioreceptor assays and *in vitro* functional assays. Radioreceptor assays have revealed that high-affinity GnRH receptors are expressed on various human tumors including breast tumors, prostate tumors, endometrial tumors, ovarian tumors, and tumors of the pancreas and liver, as well as cell lines derived from these tumors (4–9). Several investigators (6, 7, 10, 11) have shown inhibition of tumor cell growth and proliferation by GnRH agonists and antagonists in a dose- and time-dependent manner in *in vitro* studies of several cell lines. Taken together, these studies suggest that the antitumor effects of GnRH agonists and antagonists may be mediated through high-affinity receptors present on the cell membranes of tumor cells. However, this hypothesis has been questioned because several studies have failed to identify high-affinity GnRH receptors on tumors and cell lines derived from tumors (12–14).

The successful cloning of GnRH receptor from the human pituitary (15) and ovarian and breast tumors (16) enabled our use of reverse transcription-PCR, to demonstrate the expression of low levels of high-affinity GnRH receptor mRNA in a variety of extrapituitary tissues, tumors, and tumor cell lines (15, 16). A number of investigators (8, 17–19), using both reverse transcription-PCR and *in situ* hybridization techniques, also have shown the expression of GnRH receptor mRNA in various extrapituitary tissues, tumors, and tumor cell lines. These studies established that the GnRH receptor mRNA is expressed in extrapituitary tissues and tumors; however, Northern blot analysis failed to detect GnRH receptor mRNA in any of the extrapituitary tissues and tumors (15, 20). Thus, the ability of GnRH agonists and antagonists to act directly through high-affinity GnRH receptors in extrapituitary tissues and tumors has not been established because of the low levels of expression of the receptor protein. In an attempt to clarify this issue, we developed a cell line that expresses high-affinity GnRH receptor under the control of an inducible promoter. After induction of GnRH receptor expression, the cell growth and proliferation of this cell line were inhibited by GnRH agonist (D-Lys^6)GnRH and antagonist (Antide).

Materials and Methods

Development of the EcRG293 Cell Line Expressing the GnRH Receptor under the Control of an Inducible Promoter. To develop a cell line that expresses the GnRH receptor under the control of an inducible promoter, we used the ecdysone-inducible mammalian expression system from Invitrogen (Carlsbad, CA). This system is designed to allow the regulated expression of the gene of interest in mammalian cells. The expression is regulated tightly in mammalian cells with no detectable basal expression but with the capacity for a greater than 200-fold increase in expression on induction. The expression system is based on the heterodimeric ecdysone receptor of *Drosophila*. On binding of ecdysone or muristerone A, an analogue of ecdysone, the receptor activates an ecdysone-responsive promoter, which promotes high-level expression of the gene. To obtain a cell line that expresses the GnRH receptor, we excised the GnRH receptor cDNA with *Eco*RI originally cloned into a pcDNAI vector (15) and subcloned into a pIND (Invitrogen) vector. Cell line EcR293 (a human embryonic kidney cell line that constitutively expresses the ecdysone receptor) was obtained from Invitrogen. Cells were cultured in six-well plates in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 400 μg/ml zeosin, 100 units/ml penicillin, and 100 μg/ml streptomycin. The

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[3] The abbreviations used are: GnRH, gonadotropin-releasing hormone; TRH, thyrotrophic-releasing hormone.

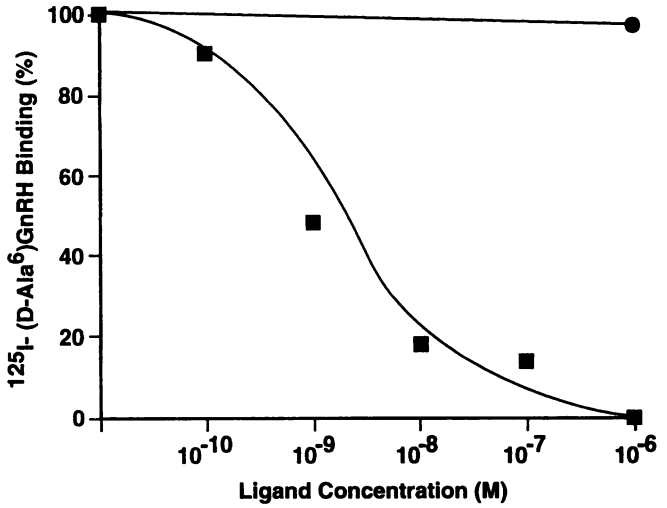

Fig. 1. Competitive displacement of ^{125}I-labeled [D-Ala^6]-des-Gly^{10}-GnRH by unlabeled ligand in a radioreceptor assay performed on membranes from muristerone A-induced EcRG293 cells (clone 10). EcRG293 (clone 10) cells were treated with muristerone A for 48 h, and cell membranes were prepared. Unlabeled [D-Ala^6]-des-Gly^{10}-GnRH was used for displacement. The results shown are the mean values derived from three independent experiments. ■, GnRH-A; ●, TRH.

cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, the cells were transfected with recombinant pIND plasmid DNA containing the GnRH receptor cDNA in sense orientation using lipofectamine (Life Technologies) as described previously (15). After 48 h, the cells were diluted 1:10 and grown on selection medium (DMEM containing 800 μg/ml G418 and 400 μg/ml zeosin) for 2 to 4 weeks; then individual colonies were isolated. Forty of the individual colonies were transferred to 48-well plates and amplified in the presence of selection medium containing G418 and zeosin. To determine the expression of the GnRH receptor, amplified colonies were plated in T75 flasks. After 24 h of plating, muristerone A was added to the cultures to a final concentration of 1 μM. The medium was replaced with fresh medium containing G418, zeosin, and muristerone A after 24 h. The expression of high-affinity GnRH receptors was determined by a radioreceptor assay 48 h after the addition of muristerone A.

Radioreceptor Assay. The cells were rinsed with cold PBS and harvested by scraping. Cell membranes were prepared for the GnRH radioreceptor assay, and the radioreceptor assay was performed as described previously (15). The radioligand used was the GnRH agonist ^{125}I-labeled [D-Ala^6]des-Gly^{10}-GnRH. Nonspecific binding was determined by the addition of unlabeled [D-Ala^6]des-Gly^{10}-GnRH (1×10^{-6} M). Clones expressing high-affinity GnRH receptors were denoted as EcRG293.

Cell Proliferation Assay. Cell proliferation was assayed using the Cell Titer 96 nonradioactive cell proliferation assay system from Promega (Madison, WI) according to the manufacturer's instructions. This assay system is based on the conversion of MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium into aqueous formazan by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan products, as measured by the absorbance at 490 nm is directly proportional to the number of living cells. This assay is comparable to the [^{3}H]thymidine incorporation assay but has the advantage that it can be performed in a 96-well plate and does not require washing, cell harvesting, or cell solubilization, and does not require the use of radioactive material. EcRG293 cells (5×10^3) were plated in 96-well plates (eight wells for each treatment) in growth medium containing G418 and zeosin. After 24 h of plating, GnRH receptor expression was induced by treating the cells with muristerone A to a final concentration of 1 μM. Twenty-four h after the addition of muristerone A, the medium was replaced with fresh medium containing G418, zeosin, and muristerone A, together with either GnRH agonist (D-Lys^6)GnRH (1 μM) or GnRH antagonist (Antide; 1 μM). TRH (1 μM) was used as a specificity control. The medium was replaced with fresh medium at 24 h intervals. At each time point, 20 μl of dye solution from the kit was added to each well. After 1 h of incubation at 37°C, the contents of the wells were mixed, and the absorbance at 490 nm was recorded using an ELISA plate reader.

Results and Discussion

On treatment with muristerone A, 2 of 40 individual clones (clones 5 and 10) exhibited expression of the high-affinity GnRH receptor. Because clone 10 expressed higher levels of GnRH receptor than clone 5, we used clone 10 for our subsequent studies. As shown in Fig. 1, treatment of EcRG293 (clone 10) cells with muristerone A to a final concentration of 1 μM resulted in the expression of high-affinity GnRH receptors. The binding affinity (IC_{50}) for the GnRH analogue (D-Ala^6)GnRH was 3 nM. This binding was specific as addition of

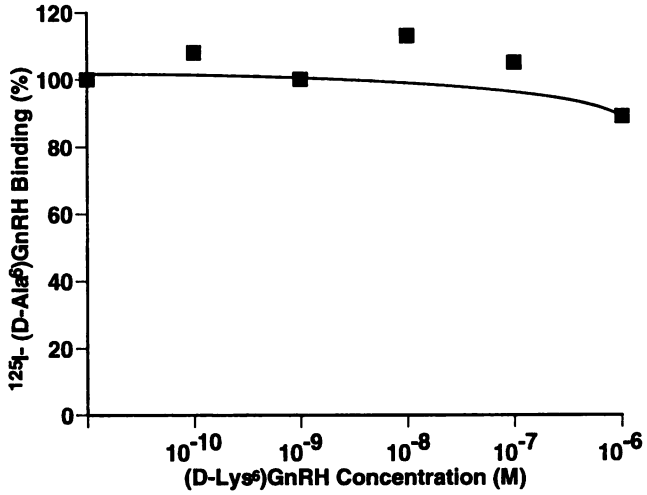

Fig. 2. Competitive displacement of ^{125}I-labeled [D-Ala^6]-des-Gly^{10}-GnRH by unlabeled ligand in a radioreceptor assay performed on membranes from uninduced EcRG293 cells (clone 10). EcRG293 (clone 10, uninduced) cells membranes were prepared. Unlabeled [D-Ala^6]-des-Gly^{10}-GnRH was used for displacement. The results shown are the mean values derived from three independent experiments.

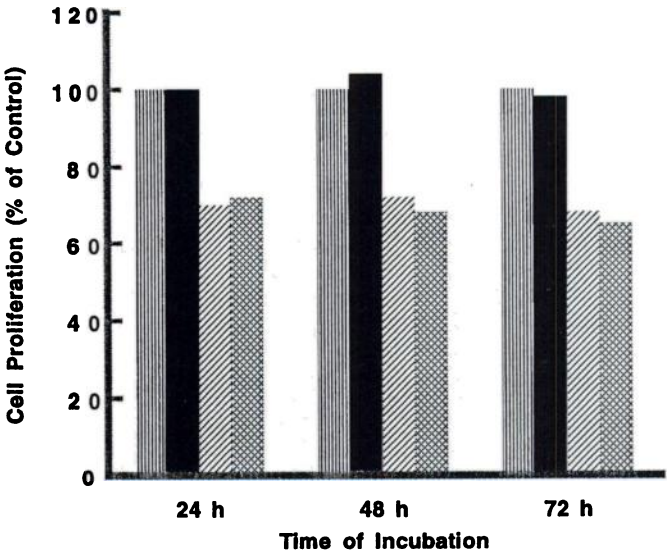

Fig. 3. Effects of GnRH agonist (D-Lys^6)GnRH and antagonist (Antide) and specificity control (TRH) on cell proliferation of EcRG293 cells expressing high-affinity GnRH receptors. EcRG293 (clone 10) cells in log phase were plated in 96-well plates. After 24 h of addition of muristerone A to the cultures, the cells were treated with GnRH agonist (1 μM D-Lys^6-GnRH, ▨), 1 μM Antide (■), or 1 μM TRH (■) for 24 to 72 h. Hormones were solubilized in DMSO with the final concentration of DMSO in cultures being 0.01%. Control cells (■) received DMSO only. Medium was replaced with fresh medium containing muristerone A and hormones every 24 h. Proliferation was assayed using a nonradioactive cell proliferation assay kit (Promega), and at each time point, 20 μl of dye solution from the kit was added to the appropriate wells. After 1 h of incubation at 37°C, the contents were mixed, and absorbance at 490 nm was recorded using an ELISA reader. Cell proliferation is expressed as % of control. Each value represents the mean of three independent experiments.

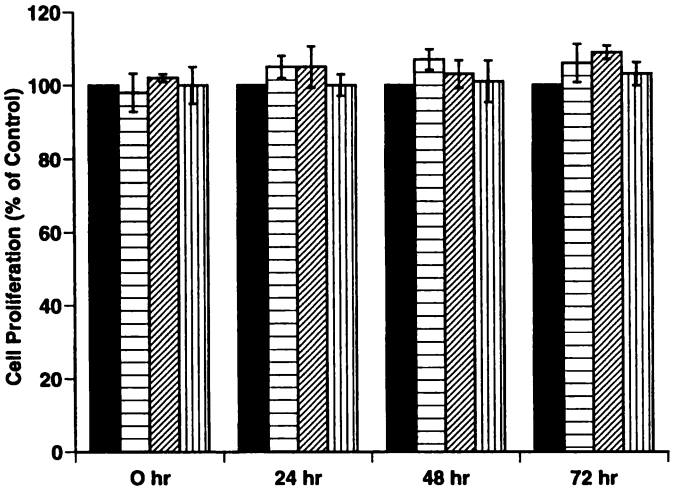

Fig. 4. Effect of GnRH agonist (D-Lys^6)GnRH and antagonist (Antide) on proliferation of uninduced EcRG293 cells. EcRG293 (clone 10) cells in log phase were plated in 96-well plates. After 24 h of plating, cells were treated with 1 μM D-Lys^6-GnRH (▨), 1 μM Antide (■), or 1 μM TRH (▩) for 24–72 h. The proliferation assay was performed as described in Fig. 3. Cell proliferation is expressed as % of control. Bars, SE (n = 5). ■, control.

TRH up to a concentration as high as 1 μM did not affect the binding of the radiolabeled ligand (Fig. 1). The binding affinity of the ligand (IC_{50} = 3 nM) is similar to that reported for the human pituitary GnRH receptor and cloned human pituitary GnRH receptor when expressed in COS-7 cells (15). Uninduced EcRG293 cells did not bind the labeled GnRH agonist (Fig. 2), indicating that the EcRG293 cells do not express GnRH receptors unless treated with muristerone A. These data suggest strongly that the GnRH receptors that are expressed on EcRG293 (clone 10) after induction with muristerone A display characteristics similar to those of the native high-affinity GnRH present in human pituitary.

To demonstrate that the effects of GnRH agonist and antagonist on tumor cells are direct and mediated through high-affinity GnRH receptors, we studied the effects of GnRH agonist (D-Lys^6)GnRH and antagonist (Antide) on EcRG293 (clone 10) cell growth and proliferation. EcRG293 (clone 10) cells (5×10^3) in log phase were plated in 96-well plates and treated with muristerone A to induce GnRH receptors expression. After 24 h of treatment with muristerone A, the medium was replaced with fresh medium containing muristerone A, and GnRH agonist (D-Lys^6)GnRH, GnRH antagonist (Antide), or TRH and incubated for 24 to 72 h. As shown in Fig. 3, the addition of 1 μM of (D-Lys^6)GnRH or Antide to the cultures resulted in a 25–30% reduction in cell proliferation at 24 h compared with control cells. This level of inhibition was retained for at least 72 h. Similar results were obtained when lower doses (10^{-9} M) of (D-Lys^6)GnRH or Antide were used (results not shown). This effect was specific because treatment with TRH did not affect cell proliferation (Fig. 3). Moreover, addition of GnRH agonist or antagonist to uninduced EcRG293 (cells not treated with muristerone A) cells did not affect cell proliferation (Fig. 4). The extent of inhibition of proliferation of EcRG293 (expressing high-affinity GnRH receptors) by GnRH agonist and antagonist (Fig. 3) were similar to those that have been reported for the inhibition of proliferation of various tumor cells on treatment with GnRH agonists and antagonist (6, 7, 10, 11). These results suggest strongly that the effects of GnRH agonists and antagonists on the growth and proliferation of cells of extrapituitary origin are specific, direct, and mediated through the high-affinity GnRH receptors present on cell membranes. However, the mechanism by which GnRH agonists and antagonists inhibit tumor cell proliferation is not known. We hypothesize that the continuous presence of agonists causes antiproliferative effects by binding to receptor, resulting in desensitization and internalization; whereas, the antiproliferative effects of antagonist are achieved by binding to receptor, resulting in termination of receptor function. Furthermore, our results suggest the possibility of using GnRH analogues as a new approach for the endocrine therapy of hormone-dependent cancers.

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