EXPERIMENTAL STUDY

LHRH might act as a negative autocrine regulator of proliferation of human ovarian cancer

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Abstract

Objective: More than 80% of human ovarian cancers express LHRH and its receptor. The proliferation of human ovarian cancer cell lines is reduced by both LHRH agonists and antagonists. This study was designed to further clarify the possible biological function of this LHRH system.

Design: As LHRH agonists and antagonists uniformly reduce proliferation of human ovarian cancer in a dose-dependent way, the effect of low concentrations of authentic LHRH was studied. In addition, longer periods of treatment (up to 9 days) were analyzed. To assess the physiological role of LHRH produced by ovarian cancer cells it was neutralized by adequate concentrations of a specific LHRH antiserum.

Methods: Human ovarian cancer cells EFO-21 and EFO-27, which express LHRH and its receptor, were incubated for 1–9 days with increasing concentrations (1 pmol/l to 10 μmol/l) of authentic LHRH or with concentrations of LHRH antiserum capable of neutralizing at least 1 nmol/l LHRH. Proliferation was assessed by counting cells.

Results and conclusions: Authentic LHRH reduced time- and dose-dependently proliferation (by maximally mean ± S.E.M. 32.7 ± 4.4%, Newman-Keuls, P < 0.001) of both ovarian cancer cell lines. At very low concentrations (1 pmol/l) a marginal reduction of proliferation or no effect was observed. A mitogenic effect of authentic LHRH was never detected. Treatment of ovarian cancer cell cultures with antiserum to LHRH significantly increased (up to mean ± S.E.M. 121.0 ± 2.8% of controls, Newman-Keuls P < 0.001) proliferation of EFO-21 and EFO-27 cells. These findings suggest that LHRH produced by human ovarian cancer cells might act as a negative autocrine regulator of proliferation.

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Introduction

In addition to its function as a key hormone in the regulation of the pituitary–gonadal axis, luteinizing hormone-releasing hormone (LHRH), also called gonadotropin releasing hormone (GnRH), probably affects a variety of human extrapituitary tissues (1–3). In a series of recent studies, it could be demonstrated that most ovarian cancer cell lines and primary tumors (>90%) express LHRH immuno- and bioactivity as well as the mRNA for LHRH (4, 5). In addition, specific high affinity binding sites for LHRH and the expression of the mRNA for the pituitary LHRH receptor have been detected in ovarian cancer cell lines and in over 80% of biopsy specimens of these cancers (5–8). The function of the expression of LHRH and its receptor is still unclear.

The proliferation of human ovarian cancer cell lines which express LHRH receptors was inhibited by both agonistic and antagonistic analogues of LHRH. These antiproliferative effects were evident at nanomolar concentrations of the LHRH analogues, suggesting that they are mediated through the LHRH receptors on the tumor cells (9–11). The exact mechanism of action of this antiproliferative effect is still obscure.

The antiproliferative effects of LHRH analogues might be explained by the following hypotheses:

1. LHRH produced by tumor cells acts as an autocrine stimulator. This effect will be blocked by potent LHRH agonists such as Triptorelin via downregulation of LHRH receptors and desensitization of their signaling mechanisms or by a competitive blockade of the receptors by LHRH antagonists.

2. LHRH produced by tumor cells acts as a negative autocrine regulator. LHRH agonists will do the same, but more effectively than authentic LHRH.

In most cell lines LHRH agonists as well as LHRH antagonists inhibit tumor growth, like the ovarian cancer cell line EFO-21 (9–11), but in a few cell lines LHRH antagonists have no effects on cell proliferation, as in the ovarian cancer cell line EFO-27 (10).
Therefore LHRH antagonists are not useful tools to elucidate the role of LHRH produced by the tumor cells as they behave like LHRH agonists in the majority of tumor cell lines, including ovarian cancer cell lines (10–13).

The present study was designed:

1. To assess the effects of authentic LHRH in a broad range of doses, including low concentrations (pmol/l) and over long time periods to check if under these conditions a stimulatory effect can be observed.

2. To neutralize LHRH produced by tumor cells with an anti-LHRH antiserum to assess the effects of LHRH produced by the tumor cells.

For this study ovarian cancer cell lines EFO-21 and EFO-27 were selected as a model system, because these cell lines have been shown to express LHRH and its receptor and their proliferation is significantly reduced by nanomolar concentrations of LHRH analogues (5, 10).

**Methods**

**Cell lines and culture conditions**

The human ovarian cell lines used were derived from a poorly differentiated serous adenocarcinoma (EFO-21) (14) or a mucinous papillary adenocarcinoma of intermediate differentiation (EFO-27) (15). The cells were cultured at 37°C in a water saturated atmosphere of 5% CO₂ in air. The medium used was based on Minimal Essential Medium (MEM, Eagle) and has been described in detail previously (10).

**Time course and dose–response proliferation experiments using authentic LHRH**

Aliquots of 2500 cells of either EFO-21 or EFO-27 cell lines were plated in multiple four-well cluster dishes of 16 mm diameter (Nunc, Roskilde, Denmark) in 1 ml of medium. After 24 h, the cells had attached to the dishes. The medium was replaced by fresh medium, and appropriate dilutions of authentic LHRH in PBS (20 µl) containing BSA (2 g/l) were added, resulting in final LHRH concentrations of 1 pmol/l to 10 µmol/l. Controls received 20 µl PBS/BSA. After 1, 3, 5, 7 and 9 days of incubation, the cells from four plates of every LHRH dose and from the respective controls were detached with 1 ml of a solution containing 0.5 g trypsin (Biochrom) and 5 mmol EDTA in 1 liter PBS/BSA. Viable cells, determined by trypan blue exclusion, were counted in a Neubauer hemocytometer (Brandt, Wertheim, Germany). In each experiment, the number of spontaneously detached cells floating in the medium and dead (trypan blue-stained) cells was assessed (for details see ref. 10). All proliferation experiments were performed in quadruplicate and reproduced at least twice in different passages of the cell lines.

**Characterization of anti-LHRH antiserum**

An LHRH RIA was performed under cell culture conditions to analyze the dilution of rabbit anti-human LHRH antiserum (Sigma, Deisenhofen, Germany) sufficient to neutralize LHRH under these conditions. The antiserum was specific for LHRH and did not react with luteinizing hormone (LH), follicle-stimulating hormone (FSH) or prolactin (16). To exclude unspecific binding, rabbit normal serum was used in control experiments. A standard curve was prepared with LHRH in a serial dilution of 1:2 from 5000 pg/ml up to 19.5 pg/ml. Rabbit anti-human LHRH antiserum was diluted in culture medium including 0.1% rabbit normal serum to final dilutions of 1:400, 1:800 and 1:1600. The specific activity of the labeled (3-125I-iodytrosyl5)-LHRH (Amersham, Braunschweig, Germany) specified by the supplier was 1585 µCi/µg. After incubation under cell culture conditions (37°C) for 2 h, the 125I-LHRH–anti-LHRH antibody complex was precipitated using a donkey anti-rabbit antibody diluted in 5% polyethylene glycol/PBS including 7% human normal serum. The precipitated complex was measured after centrifugation by gamma counting.

Mathematical analysis of binding affinity (Kₐ) and binding capacity (Bₘₐₓ) of the anti-LHRH antiserum were performed using the LiGAND program, kindly provided by Drs P J Munson and D Rodbard, Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland, USA.

**Standardization of anti-LHRH antiserum by rat pituitary bioassay**

A rat pituitary bioassay was performed to analyze whether or not the rabbit anti-human LHRH antiserum is able to inhibit the LHRH-induced LH secretion of pituitary cells under cell culture conditions. Primary rat pituitary cell cultures were prepared as described previously (17, 18) and plated in multiple four-well cluster dishes of 16 mm diameter (Nunc) in 1 ml of medium. After 24 h, the cells had attached to the dishes. The medium was replaced by fresh medium, and the cells were incubated for 3 h with appropriate dilutions of rabbit anti-LHRH serum, resulting in final antiserum dilutions of 1:10, 1:20, 1:50 and 1:100 in the absence or presence of 1 nmol/l LHRH. The LH content of the medium was determined by RIA described previously (19).

**Time course proliferation experiments using an antiserum against authentic LHRH**

Aliquots of 2500 cells of either EFO-21 or EFO-27 cell lines were plated and allowed to attach to the wells as described above. The medium was replaced by fresh medium, and the rabbit anti-LHRH antiserum which
had been tested for quantitative activity (see above) and reconstituted in 1 ml of PBS containing BSA (2 g/l) was added, resulting in a 1:40 dilution. Controls received a PBS/BSA solution or PBS/BSA containing rabbit normal serum. After 1, 3, 5, 7 and 9 days of incubation, the cells were detached and counted as described above. All proliferation experiments were performed in quadruplicate and reproduced at least twice in different passages of the cell lines.

Statistical analysis

All experiments were performed at least three times in different passages of the respective cell lines. Results are expressed as percentages of respective controls and were pooled before they were tested by one-way ANOVA followed by a Newman Keuls’ test for the comparison of individual groups, after a Bartlett test had shown that variances were homogeneous. Results are given as means ± S.E.M.

Results

Effects of authentic LHRH on proliferation of the human ovarian cancer cell lines

After 1 day of treatment with authentic LHRH (1 nmol/l) a marginal antiproliferative effect was observed (9.6 ± 2.7%, data not shown). Longer treatment or higher concentrations of authentic LHRH resulted in an increasing antiproliferative effect. On day 3, the antiproliferative effect of 1 nmol/l authentic LHRH and higher (100 nmol/l, 10 μmol/l) was significant (14.5 ± 2.8%, 16.1 ± 2.7% and 26.7 ± 4.3% respectively, P < 0.01). On day 5, even the antiproliferative effect of 10 pmol/l authentic LHRH was highly significant (16.4 ± 3.3%, P < 0.001).

Figure 1 shows the antiproliferative effects of LHRH on day 7. When human ovarian cancer cell lines EFO-21 and EFO-27 were treated with 10 pmol/l of authentic LHRH, a significant growth inhibitory effect of 17.8 ± 3.5% was observed. Treatment with a concentration of 10 μmol/l resulted in an antiproliferative effect of 32.7 ± 4.4% (P < 0.001).

A growth-promoting effect of LHRH was never observed in these experiments.

Testing of anti-LHRH antiserum under cell culture conditions via LHRH RIA

An antiserum dilution of 1:400 resulted in a maximal antiserum binding capacity of 1.16 · 10¹¹ mol/l and a binding affinity of 4.3 · 10⁹ l/mol. Bmax of an 1:800 dilution was 4.47 · 10¹¹ mol/l, while the Kd was similar to that above (Kd = 5.48 · 10⁹ l/mol). An 1:1600 dilution resulted again in an approximately 50% decrease of Bmax (2.8 · 10¹¹), while the Kd was similar to that found at the other antiserum dilutions (Kd = 4.13 · 10⁹ l/mol).

Characterization of anti-LHRH antiserum

The neutralizing effect of anti-LHRH antiserum on exogenous LHRH action in rat pituitary cell culture, shown as decreased LH secretion, was demonstrated (data not shown). LHRH at 1 nmol/l induced a threefold increase of LH secretion which was significantly reduced by several antiserum dilutions. In a dilution of 1:50, LHRH-induced LH secretion was reduced to basal levels (LH basal level: 9.0 ± 0.1 ng/ml per 3 h; antiserum reduced level: 10.7 ± 0.3 ng/ml per 3 h). An antiserum dilution of 1:10 reduced the LH secretion induced by 1 nmol/l LHRH to 5.1 ± 1.1%.

In proliferation experiments, the anti-LHRH antiserum was used in a 1:40 dilution with a calculated neutralizing capacity of 10 pmol/l LHRH, which ought to be high enough to eliminate LHRH produced by the tumor cells (5).

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Effects of anti-LHRH antiserum on cell proliferation

When EFO-21 and EFO-27 human ovarian cancer cell lines were incubated with an 1:40 dilution of the anti-LHRH antiserum, an increased proliferation was observed (Figs 2 and 3). On day 3 of antiserum treatment, a non-significant effect (103.6 ± 3.7%) as compared with controls (100%) was observed in the cell line EFO-21 which proliferates slowly (Fig. 2). On day 5 the growth-promoting effects of the LHRH antiserum were highly significant (121.0 ± 2.8%) in comparison with control cultures treated with PBS in the absence or presence of normal rabbit serum (P < 0.001). Normal rabbit serum had no mitogenic effect. Cell death in the different experiments using anti-LHRH antiserum or PBS with or without normal rabbit serum was in the same range (5%).

In the quickly proliferating cell line EFO-27 (Fig. 3) anti-LHRH antiserum induced growth acceleration was already observed on day 1 of treatment (119 ± 2.7%). This effect was highly significant in comparison with control cultures treated with PBS in the absence or presence of normal rabbit serum (P < 0.001). On day 7 of culture the mitogenic effect of LHRH antiserum vanished due to confluence of cells in both control and experimental cultures. Cell death in all experiments including the controls was in the same range (5%). A growth-promoting effect of normal rabbit serum was never observed in these experiments.

Discussion

In view of the apparent similarity of LHRH receptors in ovarian cancers to those in the pituitary, it seems reasonable to speculate that also LHRH signal transduction pathways in the tumors might be the same as those operating in pituitary gonadotrophs (20). Our findings, however, suggest that the classical LHRH receptor signal transduction pathways, known to operate in pituitary gonadotrophs, are not involved in the mediation of antiproliferative effects of LHRH analogues in ovarian cancer cells (21). LHRH analogues interfere with the mitogenic signal transduction pathway of growth factor receptors and related oncogene products associated with tyrosine kinase activity (13). Our group performed extensive studies in human ovarian cancer cell lines (EFO-21 and EFO-27). These cell lines express high affinity LHRH receptors as well as bioactive LHRH, and their proliferation is inhibited by LHRH analogues (5, 10, 12) demonstrating the existence of several components essential for a local regulatory system based on LHRH. In prostatic cancer cells, LHRH agonists inhibit proliferation by interfering with some of the cellular mechanisms mediating the stimulatory action of the epidermal growth factor (EGF) and the insulin-like growth factor (IGF) system (22, 23). Dondi et al. (24) found that LHRH agonists exert significant and dose-dependent antiproliferative action on DU 145 prostate cancer cells. Both LHRH and its receptor are expressed in this cell line, indicating that an autocrine/paracrine LHRH loop is present in androgen-independent prostate cancer cells, and may participate in the regulation of tumor growth. Recent observations in prostate cancer suggest that the LHRH receptor seems to be coupled to the Gα(i)-cAMP signal transduction pathway, rather than to the Gα(q/11)-phospholipase C pathway.
signaling system (25). In ovarian cancer cells cAMP is not involved in LHRH signaling (21). Furthermore, the precise mechanism through which LHRH and its analogues reduce cancer cell proliferation is still unknown. Thompson et al. (9) suggested that an increase of cells in the G0/G1 phase of the cell cycle and a respective reduction of the S-phase fraction might explain this phenomena. Imai et al. (26) recently proposed that LHRH might activate the pro-apoptotic system in ovarian cancer cells. In our labs, LHRH and both agonistic and antagonistic analogues did not induce apoptosis in EFO-21 and EFO-27 cells (data not shown).

One prerequisite to accept an LHRH-based autocrine system is to detect LHRH bioactivity in the culture media of ovarian cancer cell lines. Production of LHRH by EFO-21 and EFO-27 ovarian cancer cells could be clearly demonstrated in earlier experiments (5). Despite several efforts, however, we were not able to detect LHRH activity in the media conditioned by EFO-21 and EFO-27 using a highly sensitive LHRH RIA (sensitivity: 15 fmol/ml; data not published). Similarly, Ohno et al. (4) found LHRH activity in extracts of the SK-OV-3 ovarian cancer cell line but not in conditioned media.

Our data demonstrate that pmol/l concentrations (equivalent to 1 fmol/ml) of authentic LHRH had either no effects or antiproliferative effects. No mitogenetic effect of authentic LHRH on human ovarian cancer cell lines was observed. It could be speculated that even lower LHRH concentrations (fmol/l) might have stimulatory effects on cell growth as postulated by Qayyum et al. (27) for prostatic cancer cell lines. As LHRH receptor binding affinity (Kd), however, is in the range of a few nmol/l, this seems to be not very reasonable. To rule out the possibility that fmol/l concentrations of LHRH secreted by the tumor cells stimulate their proliferation, the experiments with anti-LHRH antiserum were performed. If Qayyum’s (27) hypothesis was true, neutralization of LHRH secreted by the tumor cells should result in growth inhibition. Instead of that we observed a significant growth stimulation in both cell lines. These results are rather in agreement with our theory that LHRH produced by the tumor cells reduces their proliferation. The phenomenon that LHRH is clearly detectable in cell extracts but not in conditioned medium remains an area of speculation. It might be possible that LHRH is secreted by tumor cells in a pulsatile fashion and is rapidly degraded in the medium. Alternatively, an immediate re-uptake of LHRH by the tumor cells might prevent its detection in conditioned media. Assuming a pulsatile release of LHRH by the tumor cells, leading to short-lived fmol/l concentrations of the decapeptide in the medium, it might be speculated that continuous treatment with even pmol/l concentrations as performed by us might downregulate LHRH receptors or desensitize the system and thus blunt the physiologic mitogenic effect of pulsatile LHRH release by the tumor cells. However, our data obtained with anti-LHRH antiserum clearly argue against this speculation.

In conclusion, the data obtained with low concentrations of LHRH and those obtained with anti-LHRH antiserum rather support the concept that LHRH produced by the tumor cells has an inhibitory effect on their proliferation. The exploitation of this negative autocrine LHRH system might open new therapeutic options in cancer therapy.

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References
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