High Affinity Binding and Direct Antiproliferative Effects of LHRH Analogues in Human Ovarian Cancer Cell Lines

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ABSTRACT

Recently, specific binding sites for luteinizing hormone releasing hormone (LHRH) and its analogues have been demonstrated in biopsy samples of human epithelial ovarian cancer. Their biological significance remained obscure. In this study we ascertain whether such LHRH-binding sites are also present in the human epithelial ovarian cancer cell lines EFO-21 and EFO-27 and if they could mediate antiproliferative effects of LHRH analogues. Using [125I]-D-Trp6LHRH, a high affinity/low capacity binding site was detected in both lines: EFO-21 (Kd = 1.5 x 10^-9 M; binding capacity (Bmax) = 4.9 fmol/10^6 cells) and EFO-27 (Kd = 1.7 x 10^-9 M; Bmax = 3 fmol/10^6 cells). In addition, a second class of low affinity/high capacity binding sites (EFO-21: Kd = 7.5 x 10^-6 M; Bmax = 24 pmol/10^6 cells; EFO-27: Kd = 4.3 x 10^-7 M; Bmax = 14.5 pmol/10^6 cells) was demonstrated. Specific binding of [125I]-D-Trp6LHRH was displaced with nearly equal efficiency by unlabeled [D-Trp6]LHRH, the LHRH-antagonists SB-75 and Hoe-013, and by native LHRH but not by unrelated peptides such as oxytocin and somatostatin. In the presence of 10^-9 M agonist [D-Trp6]LHRH, the proliferation of both cell lines was significantly reduced to 77% of controls after 24 h and to approx. 60% after 6 days. Lower concentrations (10^-7 M) of the agonist, significantly decreased [3H]-thymidine incorporation to 87.5% for EFO-21 and 86% for EFO-27 after 6 days. These antiproliferative effects were enhanced by increasing doses of [D-Trp6]LHRH and were maximal at 10^-5 M (EFO-21: 65.5% of control, EFO-27: 68% of control). Similar dose-dependent antiproliferative effects were obtained in EFO-21 line with the LHRH-antagonists SB-75 and Hoe-013, while these analogues had no effects on the proliferation of EFO-27 cells. SB-75 partly antagonized the antiproliferative effect of [D-Trp6]LHRH in a dose dependent way in the EFO-27 line. These data suggest that LHRH analogues can directly inhibit the in vitro proliferation of human ovarian cancer cells. This effect might be mediated through the high affinity LHRH binding sites.

INTRODUCTION

In addition to its function as a key hormone in the regulation of pituitary-gonadal axis, LHRH* probably also affects human extrapituitary tissues. In the placenta, LHRH binding sites, LHRH itself, mRNA for LHRH, and biological functions of this decapeptide have been demonstrated (for reviews, see Refs. 1 and 2). In breast and prostatic cancer, LHRH binding sites and LHRH immunoreactivity have also been shown (for reviews, see Refs. 3–5). Analogues of LHRH reduce the proliferation of some breast and prostate cancer cell lines (for reviews, see Refs. 3 and 5–8) and activate LHRH signal transduction mechanisms in breast cancer cells (8).

We have demonstrated the existence of a binding site specific for LHRH and its analogues in about 80% of epithelial ovarian cancers (9). These LHRH binding sites were of low binding affinity and high capacity (9) as in other human extrapituitary tissues including placenta (10) and breast cancer (3) and had a molecular mass of approximately 63 kDa (11). Recently, a second class of high affinity/low capacity LHRH binding sites has been described in breast (4), prostate (5), and ovarian cancer2 with binding characteristics comparable to pituitary LHRH receptors. The functional role of the LHRH binding sites in human epithelial ovarian cancer is still obscure. To ascertain if they might have biological importance, the following experiments were performed. First, we attempted to determine if two well characterized human epithelial ovarian cancer cell lines, EFO-21 and EFO-27 (12, 13), have low or high affinity LHRH binding sites. Then we determined if the potent LHRH agonist [D-Trp6]LHRH at high concentrations has an effect on the proliferation of these cancer cells. Further, we studied the time dependence and the dose-response relationship of the effects of this LHRH agonist and of two modern antagonistic analogues on the proliferation of the tumor cells. Finally, possible interactions between the agonist [D-Trp6]LHRH and the antagonist SB-75 were examined.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human ovarian cancer cell lines used have been derived from a poorly differentiated serous adenocarcinoma (EFO-21) (12) or a mucinous papillary adenocarcinoma of intermediate differentiation (EFO-27) (13) and have been described in detail previously (12, 13). The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air.

The medium used was based on Earle’s salts and contained 2-fold concentrations of Earle’s minimal essential medium, vitamins, essential and nonessential amino acids (Biochrom, Berlin, Germany). Other components were 2.2 g NaHCO3/liter (Biochrom), 4 mM L-glutamine (Merck, Darmstadt, Germany), 1 mM sodium pyruvate, 5 mg fetuin/liter, 2.5 mg transferrin/liter (Sigma, Deisenhofen, Germany), 0.25 μg glycyl-l-histidyl-l-lysine acetate (Serva, Heidelberg, Germany), and 67 mg gentamicin sulfate/liter (Biochrom). The medium was supplemented with 40 international units insulin/liter (Hoechst-Behring, Frankfurt, Germany), 10 mM 3’,5-tri-iodothyronine (Sigma) and 10% fetal calf serum (Boehringer, Mannheim, Germany).

LHRH Analogues and Other Peptides

The LHRH agonist [D-Trp6]LHRH (Triptorelin) was kindly provided by Ferring Arzneimittel, Kiel, Germany. The LHRH antagonist analogues SB-75 (Cetrorelix) ([Ac-D-Nal(2), D-Phe(4 Cl)2, D-Pal(3)3, D-Cit6, D-Ala6, D-Aga2]-LHRH) and Hoe-013 (Ramorelix), (Ac-D-Nal(2), D-Phe(4 Cl)2, D-Pal(3)3, D-Cit6, D-Ala6, D-Aga2)-LHRH) were prepared as described previously (14, 15). The LHRH antagonist

Received 12/11/92; accepted 9/15/93.

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1 Dedicated to Professor Dr. H. Maass, President of the German Cancer Society, on the occasion of his 65th birthday.

2 This study was supported by the foundations P. E. Kempkes and A. and U. Kulemann, Marburg, by Ferring Arzneimittel GmbH, Kiel, Germany, and in part by the Deutsche Forschungsgemeinschaft (SFB 215, B10). M. B. received a grant of the Fachbereich Humanmedizin of Philipps University, Marburg.

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4 The abbreviations used are: LHRH, luteinizing hormone releasing hormone; SB-75 (Cetrorelix), [Ac-D-Nal(2), D-Phe(4 Cl)2, D-Pal(3)3, D-Cit6, D-Ala6, D-Aga2]-LHRH; Hoe-013, (Ramorelix), (Ac-D-Nal-D-pcl)-Phe-D-Trp-Ser-Tyr-D-Ser(Rha)-Leu-Arg-Pro-Azagly-NH2); PBS, phosphate buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; Bmax, binding capacity.

5 G. Srkalovic, A. V. Schally, J. L. Wittliff, T. G. Day, Jr., and E. L. Jenison, Presence and characteristics of receptors for [D-Trp6]-luteinizing hormone releasing hormone and epidermal growth factor in human ovarian carcinoma, manuscript in preparation.
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Hoe-013 (Ac-D-Nal-D-(pC)-Phe-D-Trp-Ser-Tyr-D-Ser(Rha)-Leu-Arg-Pro-Azagly-NH2) (Ramorelix) was kindly provided by Dr. H. H. Sedlacek, Behringwerke, Marburg, Germany.

LHRH, oxytocin, and somatostatin were obtained from Sigma.

LHRH Radioreceptor Assay

[D-Trp6]LHRH was labeled with 125I using the lactoperoxidase method according to Clayton et al. (16) and purified on a 50 x 2.5 cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) using 0.1 M aqueous acetic acid, containing 2.5 g BSA/liter as eluent.

The specific radioactivity of the labeled peptide, determined by self-displacement in a pituitary membrane receptor assay, was 0.8 mCi/µg. The maximal tracer bindability, determined with an excess of rat pituitary membranes, was 35%. Cells grown as described above were washed with PBS containing 2 g BSA/liter and then gently removed from the culture flasks by incubation with PBS/BSA containing 0.2 g/liter EDTA (Biochrom). Cells were collected by centrifugation at 200 x g and washed twice with PBS/BSA. After aliquots were counted, cells were suspended and homogenized using a glass/glass Potter homogenizer in 10 mM Tris/HCl buffer, pH 7.6, containing 2 g BSA/liter, 2 g NaN3/liter, and 1 mM dithiothreitol (Merck). After the removal of nuclei and debris by a centrifugation step at 200 x g, plasma membranes were collected at 70,000 x g.

Aliquots of the membrane preparations, equivalent to 300,000-600,000 cells, were incubated with 4 x 10^-10 M [125I]D-Trp6-LHRH in the absence or presence of increasing concentrations (10^-11 M to 10^-4 M) of the unlabeled agonist or other peptides for 90 min at 0-4°C in a total volume of 300 µl of buffer. The incubation was stopped by dilution with 3 ml of ice-cold PBS/BSA and immediate filtration through Whatman GF/B filters pre-coated with 20 g BSA/liter PBS. Filters were then washed twice with 3 ml of PBS containing 2 g BSA/liter and then counted in a gamma spectrometer. Intrassay variance was 20-106% for the parameters calculated by the ligand program; interassay variance was between 20 and 68%. Total binding of [125I]LHRH was 6-7%, and nonspecific binding, determined in the presence of 10^-4 M [D-Trp6]-LHRH, was 2.7-3.6% of total radioactivity. Nonspecific binding to the filters in the absence of membranes was 2.3-3.1% of total radioactivity, which is always lower than nonspecific binding of membranes in the presence of 10^-4 M [D-Trp6]-LHRH, and was not influenced by the addition of unlabeled [D-Trp6]-LHRH even at 10^-4 M concentrations. Pilot studies on the incubation time had revealed that steady state was consistently achieved after 90 min under the conditions described above. Mathematical analyses of the binding data were performed using the "ligand" program (16), kindly provided by Dr. P. J. Munson and Dr. D. Rodbard, Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, NIH, Bethesda, MD.

Proliferation Assays

Time Course Experiments. Aliquots of 20,000 cells of either EFO-21 or EFO-27 were plated in multiple 4-well cluster dishes 16 mm in diameter (Nunc, Roskilde, Denmark) in 1 ml of medium (see above). After 24 h the cells had attached to the dishes. Medium was replaced by fresh medium and either 20 µl of a solution of [D-Trp6]-LHRH (5 x 10^-4 M) in PBS/BSA, resulting in a final LHRH agonist concentration of 10^-5 M, or 20 µl of PBS/BSA (controls) were added. Under these conditions almost complete confluency of cells was reached within 2 days after addition of the medium.

These additions of LHRH analogue or vehicle were repeated every 24 h for 5 days. Every day cells from 1 dish (4 wells) each of the [D-Trp6]-LHRH or the control group were detached with 1 ml of 0.5 g Trypsin (Biochrom) and 5 mmol EDTA (Biochrom) in 1 liter of PBS/BSA. Viable cells determined on the basis of Trypan blue exclusion were counted in a Neubauer hemocytometer. In control group were detached with 1 ml of 0.5 g Trypsin (Biochrom) and 5 mmol EDTA (Biochrom) in 1 liter of PBS/BSA. Viable cells determined on the basis of Trypan blue exclusion were counted in a Neubauer hemocytometer. Every day cells from 1 dish (4 wells) each of the [D-Trp6]-LHRH or the medium was changed and 20 µl of PBS/BSA or appropriate dilutions of [D-Trp6]-LHRH, SB-75, or Hoe-013 were added, resulting in final analogue concentrations of 10^-11 M to 10^-5 M. After 3 days the medium was replaced by fresh medium containing LHRH analogues. After another 3 days of incubation, cells were detached and counted as described above.

Interactions between [D-Trp6]-LHRH and SB-75. Aliquots of 20,000 cells of EFO-27 line were incubated for 6 days in the absence or presence of increasing concentrations (10^-11 M to 10^-5 M) of [D-Trp6]-LHRH as described above. In addition the cells were simultaneously exposed to either 10^-3 M SB-75, or 10^-5 M SB-75, or vehicle starting 2 h before the treatment with [D-Trp6]-LHRH.

All proliferation experiments were performed in quadruplicates and reproduced twice in different passages of the cell lines.

Radioimmunoassay of [D-Trp6]-LHRH

[D-Trp6]-LHRH in the culture medium was measured after appropriate dilution as previously described (17) using standards prepared with diluted medium.

Statistical Analyses

Cell numbers in the individual time course experiments were tested for significant differences using a Mann-Whitney U test. In addition, the data obtained in 3 independent experiments with each cell line were expressed in percentages of the respective controls (PBS/BSA = 100%) and pooled before they were analyzed with a Mann-Whitney U test.

The data from the dose-response experiments and on the interactions of [D-Trp6]-LHRH and SB-75 in EFO-27 line were expressed as percentages of the controls (PBS/BSA = 100%). The data from 3 independent experiments, run in 3 different passages of the cell lines, were tested for significant differences by one-way analysis of variance followed by a Newman-Keuls' test after a Bartlett test had shown that variances were homogenous.

RESULTS

[D-Trp6]-LHRH Binding Sites

Specific binding of [125I]D-Trp6-LHRH in EFO-21 and EFO-27 lines was nearly equally well displaced by unlabeled [D-Trp6]-LHRH, the LHRH antagonists SB-75 and Hoe-013, and by native LHRH. Peptides unrelated to LHRH such as oxytocin and somatostatin did not affect the binding of [125I]D-Trp6-LHRH (Fig. 1). Analysis of the binding data obtained with [D-Trp6]-LHRH in the two cell lines with the Ligand program revealed the existence of 2 different classes of binding sites in each cell line. One site shown was with high binding affinity and low capacity (EFO-21: Kd = 1.5 x 10^-9 M; Bmax = 4.9 pmol/10^6 cells; EFO-27: Kd = 1.7 x 10^-9 M; Bmax = 3 fmol/10^6 cells), while the other was with low binding affinity and high capacity (EFO-21: Kd = 7.5 x 10^-6 M; Bmax = 24 pmol/10^6 cells; EFO-27: Kd = 4.3 x 10^-6 M; Bmax = 14.5 pmol/10^6 cells) (Fig. 2).

Proliferation Assays

Time Course Experiments. When 20 µl of [D-Trp6]-LHRH (5 x 10^-4 M) were added every 24 h for 6 days to the culture medium (1 ml), proliferation of both ovarian cancer cell lines was significantly reduced to 77% of that in control cultures (20 µl PBS/BSA) after 24 h. This antiproliferative effect increased gradually with time. After 6 days of incubation, cells were virtually the same in both PBS/BSA or [D-Trp6]-LHRH treated cultures.

When 20 µl of [D-Trp6]-LHRH (5 x 10^-4 M) were added to the culture medium (1 ml) only once on day 0 of the experiment, the absolute inhibition of cell proliferation was slightly less in comparison with daily addition and also showed a clear time dependence with a maximal effect after 6 days of culture (Fig. 5).
Dose-Response Experiments. The proliferation of both cell lines (EFO-21 and EFO-27) was dose dependently inhibited by 6 days of treatment with [D-Trp⁶]LHRH. At a 10⁻¹¹ M concentration, slight decreases in cell numbers to 92 ± 2.2% (EFO-21, P < 0.05) or 96.5 ± 3.7% (EFO-27) were observed. At 10⁻⁹ M concentration of the analogue, the reduction in cell numbers was significant in both lines (EFO-21: 87.5 ± 2% of control, P < 0.01; EFO-27: 86 ± 4% of control, P < 0.05). [D-Trp⁶]LHRH at 10⁻⁷ M had even greater antiproliferative effects on both cell lines. These effects became maximal at 10⁻⁵ M of the analogue (EFO-21: 65.5 ± 2.6% (P < 0.01) and EFO-27: 68 ± 3.3% of control (P < 0.01) (Fig. 6).

A similar dose-response relationship for the antiproliferative effects was observed for the LHRH antagonists SB-75 and Hoe-013 in the EFO-21 line (Fig. 7), while neither antagonist had an effect on the proliferation of EFO-27 (Fig. 8).

Interactions between [D-Trp⁶]LHRH and SB-75. In this series of experiments, treatment with [D-Trp⁶]LHRH showed the same dose dependent antiproliferative effect in the EFO-27 line as described above, while SB-75 alone (10⁻⁷ M, 10⁻⁵ M) as in the previous experiments had no effect on proliferation. When the cells were incubated simultaneously with the agonist and antagonist, the antiproliferative effect of [D-Trp⁶]LHRH was in part significantly antagonized by SB-75 in a dose dependent way (Fig. 9).

Stability of [D-Trp⁶]LHRH during Cultures

The determination of [D-Trp⁶]LHRH concentrations by radioimmunoassay in the culture medium before, during, and after 3-6 days of incubation with the cells revealed that no measurable decomposition or metabolism of the peptide occurred (data not shown).

DISCUSSION

Two types of LHRH-binding sites could be demonstrated in the human ovarian cancer cell lines EFO-21 and EFO-27. The low affinity/high capacity site had similar characteristics as the low affinity LHRH binding sites previously described in biopsy material from human epithelial ovarian cancers (9, 11), human placenta (10), breast (3), or endometrial cancer (18, 19). The second class of binding sites for LHRH found in EFO-21 and EFO-27 seems to be identical with the high affinity LHRH-binding site described by Fekete et al. (4) for breast cancer or by Skrlcovic et al. (18) for endometrial or ovarian cancer. The high affinity binding site also seems to be very similar to the rat pituitary LHRH receptor (4). [¹²⁵I, D-Trp⁶]LHRH was nearly equally well displaced by unlabeled [D-Trp⁶]LHRH, native LHRH,
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Fig. 3. The effect of [D-Trp'']LHRH on the proliferation of EFO-21 (A) and EFO-27 (B). The analogue (A) was added daily (day 0 to 5) (20 μl of a 5 × 10^{-4} M solution in PBS/BSA) to the cultures (1 ml of medium) while to control cultures (C) 20 μl of vehicle were added every day. Points, means + SE of 4 cultures. a, P = 0.014, Mann-Whitney U-test. These experiments were reproduced twice with nearly identical results in 2 different passages of the cell lines (summary in Fig. 4).

and the two LHRH antagonists SB-75 and Hoe-013. In contrast, peptides unrelated to LHRH such as oxytocin and somatostatin did not influence the binding of [{\textsuperscript{125}I, D-Trp''}]LHRH to the membranes of the two ovarian cancer cell lines. These findings indicate that the binding sites are specific for LHRH and its agonistic and antagonistic analogues.

The second series of experiments were designed to ascertain whether these LHRH binding sites could transmit effects of LHRH agonists and influence the proliferation of EFO-21 or EFO-27 cells. For this screening, we deliberately chose a high concentration (10^{-5} M) of [D-Trp'']LHRH in the range of the Kd of the low affinity binding site. Because we had no data on the chemical stability and metabolism of the analogue during incubation with the cells, [D-Trp'']LHRH was added to the cells every 24 h. For these screening experiments, we also chose optimal growth conditions for the cells (FCS, insulin, transferrin). The experiments, repeated twice in different passages of the cell lines with highly reproducible results, clearly showed a marked antiproliferative effect as early as 24 h after exposure to the LHRH agonist. This inhibition increased gradually until day 6. Analysis of the [D-Trp'']LHRH content in the medium revealed that the daily addition of the analogue led to an accumulation of the substance. The data obtained thus gave no answer if the relative decrease in cell number over this time period was due to the time factor alone or to the slight increase (factor 6) in the concentration of [D-Trp'']LHRH.

In the subsequent proliferation experiments we therefore added the analogue only once on day 0. The results obtained showed that the antiproliferative effects of [D-Trp'']LHRH were time dependent. However, the relative reduction of cell growth in these experiments after 2 to 6 days was less marked than in the first series with daily addition of the analogue. This indicates that there is a cumulative effect of increase in time and dose.

In the experiments that followed, the dose-response relationship of the antiproliferative effects of [D-Trp'']LHRH was addressed. The data obtained show that at 10^{-9} M concentration of the analogue, a significant reduction in cell numbers occurred in both cell lines. In EFO-21 a slight but significant effect was observed at 10^{-11} M of [D-Trp'']LHRH. At higher concentrations of the analogue (10^{-7} and 10^{-5} M), there was a significant gradual increase in the antiproliferative effect. When this experiment was repeated under serum free conditions, the proliferation of both cell lines in the absence of [D-Trp'']LHRH was markedly slower than in the presence of FCS and insulin. The addition of increasing concentrations (10^{-11} M to 10^{-5} M) of [D-Trp'']LHRH resulted in virtually the same significant percentage of inhibition of proliferation in both EFO-21 and EFO-27 lines, as obtained under the conditions described above (preliminary data not shown). This might indicate that the antiproliferative effects of [D-Trp'']LHRH are not exclusively due to the antagonism of the effects of insulin or growth factors in FCS. At present, detailed studies on the
Fig. 5. The effect of [D-Trp6]LHRH on the proliferation of EFO-21 (A) and EFO-27 (B). The analogue (A) (20 μl of a 5 × 10−4 M solution in PBS/BSA) was added to the cultures (1 ml of medium) only on day 0 of the experiment. To the control cultures (C), 20 μl of vehicle were added on day 0. The data were obtained in 3 independent experiments run in quadruplicate in different passages of the cell lines and are presented in the same way as in Fig. 4; a, P < 0.001, Mann-Whitney U test.

interactions between defined growth factors and LHRH-analogues under serum free conditions are performed in our laboratory, which should clarify this issue.

In the next set of experiments, the effects of two modern LHRH-antagonists were studied. In EFO-21 line, both SB-75 and Hoe-013 produced similar dose dependent antiproliferative effects as did the agonistic analogue. In EFO-27 line neither SB-75 nor Hoe-013 had any effect on the proliferation of the cells, even at the high concentration of 10−5 M.

Since the proliferation of EFO-27 cells was not affected by LHRH antagonists, we used this cell line to study if the antiproliferative effect of [D-Trp6]LHRH could be influenced by simultaneous treatment with the antagonistic analogue SB-75. In this series of experiments SB-75 partly antagonized the antiproliferative effects of 10−9 M to 10−5 M concentrations of [D-Trp6]LHRH. This antagonistic activity was clearly dose dependent but did not result in a complete inhibition of the antiproliferative effect of the agonist. Even at 10−9 M [D-Trp6]-LHRH, a 10,000-fold excess of SB-75 did not completely block the antiproliferative effect of the agonist. The difference between the cell number in controls (100 ± 0.5%) and that in cultures treated with 10−9 M [D-Trp6]-LHRH plus 10−5 M SB-75 (92 ± 1.4%) was still statistically significant (P < 0.01), although the antagonist clearly partially reduced (P < 0.01) the antiproliferative effect of 10−9 M [D-Trp6]-LHRH alone which lowered cell number to 85 ± 1% of controls. As the displacement of [125I, D-Trp6]LHRH by unlabeled [D-Trp6]LHRH and SB-75 was virtually identical and the cells were preincubated with 10−5 M SB-75 for 2 h before the agonist [D-Trp6]LHRH was added, it might be speculated that the interaction between the two LHRH analogues in this tumor cell line is not exclusively explained by a simple stoichiometric competition for the high affinity binding site. At the moment, it is still obscure whether the antiproliferative effect of the agonist analogue is due to a receptor mediated activation or inhibition of specific cellular functions or to a down-regulation of the putative receptor and/or its signal transduction mechanism (see below). Therefore, further attempts to explain the nature of the interaction between [D-Trp6]LHRH and SB-75 would be highly speculative and should be postponed until detailed analyses on their effects on receptor concentration, receptor mRNA, and on signal transduction system are available (see below).

Our data are in contrast to the results of Slotman et al. (20) who observed only small, although statistically significant antiproliferative effects of high concentrations (10−6 M) of the LHRH agonist buserelin in the ovarian cancer cell lines OVC NOVA, OV 166, and OV 1225. Comparative experiments could establish whether this discrepancy is due to differences in the potency of the LHRH analogues used, the cell lines studied, or the experimental conditions.

Thompson et al. (21) recently reported marked antiproliferative effects of high concentrations (10−6 M and 10−4 M) of the LHRH agonist leuprolide in the human epithelial ovarian cancer cell line 2774 which are in good agreement with our findings. However, lower

Fig. 6. Effects 6 days of treatment with increasing concentrations of [D-Trp6]LHRH on the proliferation of EFO-21 (A) or EFO-27 (B). Cell number is expressed as a percentage of the controls (vehicle only = 100%). Columns, means ± SE of data obtained from 3 independent experiments run in quadruplicate in 3 different passages of the cell lines. a, P < 0.01 versus C Newman-Keuls; b, P < 0.01 versus 10−11 M; c, P < 0.01 versus 10−14 M; d, P < 0.01 versus 10−6 M; e, P < 0.05 versus C; f, P < 0.01 versus 10−9 M analysis of variance, P < 0.001.
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Fig. 7. Effects of 6 days of treatment with increasing concentrations of SB-75 (A) or Hoe-013 (B) on the proliferation of EFO-21. Cell number is expressed as a percentage of the controls (vehicle only = 100%). Columns, means ± SE of data obtained from 3 independent experiments run in quadruplicate in 3 different passages of the cell lines: a, P < 0.01 versus C Newman-Keuls; b, P < 0.01 versus 10⁻¹⁹ M; c, P < 0.01 versus 10⁻¹⁹ M; d, P < 0.05 versus 10⁻¹¹ M; e, P < 0.05 versus 10⁻⁹ M; analysis of variance, P < 0.001.

concentrations (10⁻⁷ M, 10⁻⁹ M), had no effects on the proliferation of this cell line in their experiments (21). The reasons for this discrepancy as compared to our results might be the same as discussed above.

The data reported by Thompson et al. (21) and in this article provide clear evidence that agonistic analogues of LHRH can directly slow down the proliferation of human epithelial ovarian cancer cells. Since we found no increase in the number of dead cells in cultures treated with [D-Trp⁶]LHRH as compared to controls, it might be assumed that the analogue does not induce cell death or apoptosis but rather reduces the proliferation rate. This assumption is supported by the findings of Thompson et al. (21) who showed that LHRH-agonist treatment caused a reversible 5-6% increase in the portion of cells in the resting phase, G₀-G₁, compared to controls and a corresponding decrease in the portion of cells in DNA synthesis phase. Future experiments will have to show whether the same explanation applies to the cell lines used in our study. In addition, long term cultures could help to determine if LHRH analogues lead to a complete arrest at some point in the cell cycle or to a slowing of the cycle.

Our observations, that significant antiproliferative effects of [D-Trp⁶]LHRH occur at 10⁻⁹ M and in the EFO-21 line even at 10⁻¹¹ M, are in accordance with the results of our binding studies that revealed a high affinity/low capacity binding site in both cell lines. It might be reasonable to speculate that this binding site is a receptor, which mediates the antiproliferative effects of LHRH agonists.

The exact mechanism of this effect at the receptor level, however, is still obscure. It is not known whether a putative endogenous ligand stimulates proliferation of the cells through this receptor, which might be down-regulated by the continuous treatment with a potent LHRH antagonists. The findings obtained in EFO-21 line with the two LHRH antagonists confirms this view. An alternative hypothesis is that the receptor mediates direct antiproliferative effects of LHRH agonists. This speculation is supported by the findings in the EFO-27 line, in which the agonistic analogue inhibited proliferation, while both LHRH antagonists alone were ineffective and SB-75 partially antagonized the antiproliferative effect of [D-Trp⁶]LHRH. In addition, it cannot be distinguished at this time, which of the two LHRH-binding sites is relevant, especially considering the findings of Thompson et al. (21) that only high doses (10⁻⁶ M and 10⁻⁴ M) of their LHRH agonist were effective in their cell line. It appears probable that the mechanism of action is not uniform but that individual response patterns exist in different ovarian cancer cell lines. Future systematic experiments on the regulation of both LHRH binding sites and their mRNA (22) by LHRH agonists and antagonists and their interactions may answer these open questions.

Assuming that the effects of LHRH analogues in ovarian cancer cells are mediated through either LHRH binding site, the subsequent signal transduction mechanism is obscure. Future studies should elucidate whether the mechanisms activated by LHRH in the pituitary gonadotrophs such as phospholipase C, intracellular Ca²⁺ mobilization, protein kinase C (for a review, see Ref. 23) are also of importance in the mediation of the antiproliferative effects of LHRH analogues in human ovarian cancer cells. Alternatively, LHRH analogues could
activate a phosphoprotein tyrosine phosphatase, thus counteracting tyrosine kinase mediated effects of growth factors or related oncogene products, as has been demonstrated for human pancreatic cancer cells (24, 25). As mentioned above, experiments designed to study the possible interactions between LHRH analogues and growth factors under serum free conditions have been started in our laboratory.

It might be argued that the antiproliferative effects of high concentrations of LHRH analogues observed by Thompson et al. (21) and in our study are due to some nonspecific mechanism, e.g., toxic effects of D-amino acids liberated after enzymatic cleavage of the analogues. The fact that the antiproliferative effects are clearly seen at $10^{-11}$ or $10^{-9}$ M concentrations of LHRH agonists and, in case of EPO-21 line, also LHRH antagonists favors a specific receptor mediated mechanism. In addition, the finding that high concentrations ($10^{-5}$ M) of SB-75 and Hoe-013 that are highly substituted with D-amino acids did not inhibit the proliferation of EPO-27 while low concentrations ($10^{-9}$ M) of the agonistic analogue were clearly antiproliferative in these cells, argues against a nonspecific mechanism.

The natural endogenous ligand for the LHRH binding sites in ovarian cancer is still unknown. LHRH immunoreactivity has been demonstrated in breast and prostatic cancer (for reviews, see Refs. 5 and 6). Similar experiments, including the search for the respective mRNA, ought to be performed in human ovarian cancer to elucidate whether an autocrine regulation system based on LHRH or related compounds exists in this malignancy.

Even if such a natural ligand which may act as a growth factor does not exist, our findings might lead to new therapeutical approaches to epithelial ovarian cancer. Because direct antiproliferative effects of [D-Trp"]LHRH are clearly evident at $10^{-9}$ M and these concentrations are achieved in serum of patients treated with sustained delivery systems (microcapsules) of this analogue (26), [D-Trp"]LHRH should theoretically also reduce the proliferation of ovarian cancer in vivo. Several preliminary reports demonstrated a favorable response of some patients with refractory ovarian epithelial cancer to treatment with LHRH agonists (for a review, see Ref. 27). Controlled clinical trials on the efficacy of LHRH agonists in earlier stages of the disease are also being performed (for a review, see Ref. 27).

The elucidation of the mechanism by which LHRH analogues reduce the proliferation of ovarian cancer cells is important for the clinical use of these drugs because a better understanding of their mechanism of action would accelerate the development of an efficacious therapeutical regimen. Since the present methods for therapy of ovarian cancer are unsatisfactory (for a review, see Ref. 28), a successful development of a nontoxic endocrine therapy based on LHRH analogues would represent a relevant advance.

In conclusion, our data show that the proliferation of human epithelial ovarian cancer cells can be reduced by agonistic and in one cell line also by antagonistic analogues of LHRH in a time and dose dependent fashion. The tumor cells have high affinity binding sites for [D-Trp"]LHRH with a $K_d$ in the nanomolar range. The antiproliferative effects of LHRH analogues are seen at $10^{-9}$ concentrations. Therefore, we assume that these LHRH binding sites could be the receptors mediating the inhibitory effects of LHRH analogues on tumor cell proliferation.

ACKNOWLEDGMENTS

We are grateful to Dr. H. H. Sedlacek, Behringwerke, Marburg, Germany, for the gift of Hoe-013, to Drs. P. J. Munson and D. Rodbard, Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, NIH, Bethesda, MD, for providing the Ligand program and to Drs. H. Prinz and C. Schade-Brittinger Institute for Medical Biometry, Philipps University Marburg, for their help in the statistical analyses.

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LHRH ANALOGUES AND HUMAN OVARIAN CANCER CELL LINES


High Affinity Binding and Direct Antiproliferative Effects of LHRH Analogues in Human Ovarian Cancer Cell Lines

Günter Emons, Olaf Ortmann, Martin Becker, et al.