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Luteinizing hormone-releasing hormone induces JunD–DNA binding and extends cell cycle in human ovarian cancer cells

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Abstract

Expression of luteinizing hormone–releasing hormone (LHRH) and its receptor as part of an autocrine regulatory system of cell proliferation has been demonstrated in a number of human malignant tumors, including cancers of the ovary. This study was conducted to investigate whether LHRH induces activation of JunD and affects cell cycle regulation and DNA synthesis. Treatment of primary human ovarian cancer cells and human ovarian cancer cell lines EFO-21 and EFO-27 with LHRH agonist triptorelin (100 nM) resulted in an increase in $G_{0/1}$ phase and a decrease in $G_{2/S}$ phase of cell cycle. Treatment of quiescent EFO-21 or EFO-27 cells with triptorelin (100 nM) resulted in a 46.7 or 44.2-fold increase of AP-1 activation, respectively ($p < 0.001$). Maximal binding of JunD on DNA consensus sequence was found after 4 h of treatment of quiescent EFO-21 or EFO-27 cells with triptorelin (100 nM). DNA synthesis was significantly decreased to $45.5 \pm 11.4\%$ (day 0 = control = 100%; $p < 0.001$) after 3 days of triptorelin (1 nM) treatment. These results suggest that LHRH agonist triptorelin induces JunD–DNA binding, resulting in reduced proliferation as indicated by increased $G_{0/1}$ phase of cell cycle and decreased DNA synthesis. Since LHRH activates nucleus factor kappa B (NFκB) and protects ovarian cancer cells from doxorubicin-induced apoptosis and JunD is shown to decrease cell cycle and cell proliferation, we propose that JunD activated by LHRH acts as a modulator of cell proliferation and cooperates with the anti-apoptotic and anti-mitogenic functions of LHRH.

Keywords: LHRH; GnRH; Ovarian cancer; JunD; Cell cycle; DNA synthesis

The expression of luteinizing hormone–releasing hormone (LHRH) and its receptor as a part of a negative autocrine regulatory system of cell proliferation has been demonstrated in a number of human malignant tumors, including cancers of the breast, ovary, and endometrium (for review see [1]). Dose-dependent anti-proliferative effects of LHRH agonists in cell lines derived from these cancers have been observed by various investigators (for review see [1]). LHRH antagonists have also marked anti-proliferative activity in most breast, ovarian, and endometrial cancer cell lines tested, indicating that the dichotomy of LHRH agonists and antagonists might not apply to the LHRH system in cancer cells (for review see [1]). The classical LHRH receptor signal-transduction mechanisms, known to operate in the pituitary [2,3], are not involved in the mediation of anti-proliferative effects of LHRH analogs in cancer cells [4]. The LHRH receptor rather interacts with the mitogenic signal transduction of growth-factor receptors and related oncogene products associated with tyrosine kinase activity via activation of a phosphotyrosine phosphatase, resulting in downregulation of cancer cell proliferation (for review see [1]). In addition, LHRH activates nucleus factor kappa B (NFκB) and protects the cancer cells from doxorubicin-induced apoptosis [5]. Recently, we found that in endometrial cancer cells, LHRH agonist triptorelin induces activation of the c-Jun N-terminal kinase/activator protein-1 (JNK/AP-1) pathway, independently from the known AP-1 activators, protein kinase C (PKC) or mitogen-activated protein kinase (MAPK, ERK) [6]. It is well established that modulation of AP-1 activity is one of

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the most critical steps in the control of cell proliferation and transformation. It was recently reported that JunD, which is usually constitutively expressed in cells, may function as a negative regulator of cell proliferation, since overexpression of JunD slowed cell growth and resulted in an increase in the percentage of cells in G_0/G_1 phase of cell cycle [7]. It was shown that inhibition of proliferation by 1,25-dihydroxyvitamin D_3 in chronic myelogenous leukemia cells is accompanied by an increase in JunD/AP-1 activity, suggesting that JunD may play a role in the anti-proliferative actions of vitamin D_3 [8]. Bernstein and Walker reported that tumor promotion resistant cells are deficient in JunD–DNA binding and JunD expression and form different AP-1–DNA complexes than promotion sensitive cells [9]. In addition, Weitzmann et al. [10] found that JunD protects cell from p53-dependent senescence and apoptosis by acting as a modulator of the signaling pathways that link Ras to p53.

LHRH and its analogs have anti-proliferative actions on ovarian cancer cells [11] and activate NFκB and, thus, protect the cancer cells from doxorubicin-induced apoptosis [5]. In addition, NFκB is reported to functionally cooperate with JunD [12]. To assess whether LHRH induces JunD activation and slows cell cycle in ovarian cancer cells, we analyzed whether or not LHRH has effect on cell cycle, JunD–DNA binding, and DNA synthesis.

Materials and methods

Cell lines and culture conditions. Primary human ovarian cancer cells were derived from a malignant pleural effusion of a 77-year-old woman with a moderately differentiated ovarian adenocarcinoma (FIGO IV b). The cells were obtained and processed in accordance with the German ethical guidelines. The human ovarian cancer cell lines used were derived from a poorly differentiated serous adenocarcinoma (EFO-21) or a mucinous papillary adenocarcinoma of intermediate differentiation (EFO-27). The cells were cultured as described in detail previously [13]. To assess LHRH agonist triptorelin-induced AP-1 activation, cells were transfected with pAP-1-SEAP. Subsequently, these cells were cultured for 96 h in the absence of FCS and phenol red, with or without 100 nM triptorelin. Every 24 h, 200 μl of the media was collected and analyzed for SEAP activity.

Flow cytometry. To show whether or not LHRH affects cell cycle, the cells were cultured for 72 h in the absence or presence of LHRH agonist triptorelin (100 nM). A pellet containing 1×10^6 cells was gently resuspended in 500 μl hypotonic fluorochrome solution containing 0.1% Triton X-100 (Sigma, Deisenhofen, Germany), 0.1% sodium citrate (Sigma), and 50 μg/ml propidium iodide (Sigma). Cell suspensions were kept at 4 °C in the dark overnight before flow cytometry analysis of cellular DNA content was performed using a FACScan (Becton Dickinson, Mountain View, CA).

Plasmids and transfection. pAP-1-SEAP (Clontech, Palo Alto, USA) is designed to monitor the induction of AP-1 and the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) signal transduction pathway [14]. pAP-1-SEAP contains six tandem copies of the AP-1 enhancer fused to HSV-TK promotor. pTK-SEAP (Clontech) was used as a negative control to determine the background signals associated with the culture medium. The enhancerless pTK-SEAP contains HSV-TK upstream of the SEAP coding sequence. Cells (200,000) were grown for 24 h on 30-mm plates. Transfections were done using the Superfect liposome reagents and following manufacturer's instructions (Qiagen, Hilden, Germany) and cells were treated as described above. Chemiluminescence detection of SEAP activity was performed according to manufacturer's instructions (Clontech) using a plate fluorometer (Berthold, Bad Wildbach, Germany).

Extraction of nuclear proteins. Nuclear extracts from EFO-21 and EFO-27 cells treated without or with LHRH agonist triptorelin (100 nM) for 2, 4, and 24 h were isolated by a modification of the method by Schreiber et al. [15]. Briefly, treated or control cells were gently scraped from the dishes, collected by centrifugation at 750*g* for 5 min, and washed once with ice-cold HEPES buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1 mM DTT). All subsequent manipulations were carried out at 4 °C. The cells were resuspended in 1 ml ice-cold HEPES buffer with 0.4% NP-40 for 10 min and then centrifuged at 750*g* for 5 min. The cell pellet was resuspended in 100 μl of another ice-cold HEPES buffer (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT) allowed to swell for 10 min and the cells were disrupted for 30 min using a magnetic homogenizer. The nuclei were centrifuged at 7500*g* for 15 min. The supernatant was transferred into a fresh cup and stored at −80 °C. The protein concentrations were determined by the method of Bradford (Bio-Rad, München, Germany).

Electrophoretic mobility shift assay (EMSA). Samples containing 10–15 μg protein from nuclear extracts of untreated or LHRH agonist triptorelin treated cells were used in DNA-binding reactions. EMSA was performed according to manufacturer's instructions using the LightShift Chemiluminescent EMSA KIT (Pierce, Rockford, IL). A 3′-biotin end-labeled DNA containing the JunD binding site was used: 5′-CTA GAC GCT TGA TGA CTC AGC CGG AA-3′. The reaction products were separated on 6% native polyacrylamide gels in 0.5X TBE, transferred to Hybond-N^+-membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and exposed to Kodak Omat X-ray film (Eastman Kodak, Rochester, NY).

[^{3}H]thymidine incorporation. The cells were plated in 96-well dishes (Nunc, Roskilde, Denmark). The confluent cultures were washed and incubated in 200 μl serum-free medium supplemented with BSA (1 g/L), LHRH agonist triptorelin (1 nM), and [^{3}H]thymidine (85 Ci/mmol; 0.2 μCi per well, Amersham Pharmacia Biotech) for 72 h. The cells were then washed with ice-cold PBS twice, treated with 10% trichloroacetic acid (TCA) for 10 min at −20 °C, washed with 10% TCA followed by 95% ethanol, and lysed with 400 μl of 0.25 N NaOH per well. Incorporated radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Unterschleissheim, Germany).

Statistical analysis. All experiments were reproduced three times in different passages of the respective cell lines or the primary human ovarian cancer cells. Data were tested for significant differences using Mann–Whitney *U* test. Results are given as means ± SEM.

Results

Effects of LHRH agonist triptorelin on the cell cycle of ovarian cancer cells

The effects of LHRH agonist triptorelin on cell cycle of primary human ovarian cancer cells and human ovarian cancer cell lines EFO-21 and EFO-27 were measured using flow cytometry. Treatment of the primary human ovarian cancer cells with LHRH agonist triptorelin (100 nM) resulted in an increase from 68% to 79% of cells in the $G_{0/1}$ phase and a decrease from 31%

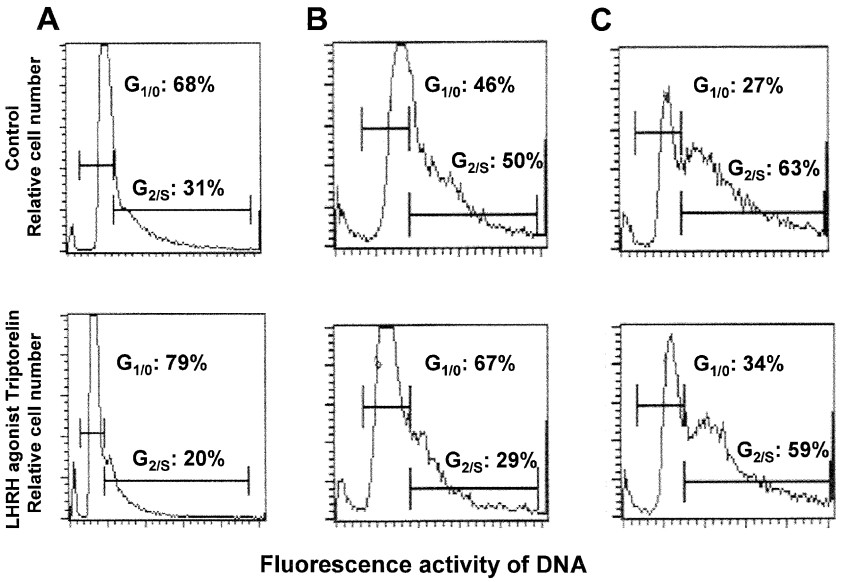

Fig. 1. Flow cytometric analysis of cell cycle distribution of primary human ovarian cancer cells derived from a malign pleural effusion of a 77-year-old woman with a moderately differentiated ovarian adenocarcinoma (FIGO IV b) (A) and ovarian cancer cell lines EFO-21 (B) and EFO-27 (C) after treatment without (control) or with LHRH agonist triptorelin (100 nM) for 72 h. The DNA histograms are representative of three independent experiments.

to 20% of cells in the $G_{2/S}$ phase of cell cycle (Fig. 1A). Treatment of EFO-21 (Fig. 1B) or EFO-27 (Fig. 1C) ovarian cancer cells with LHRH agonist triptorelin (100 nM) resulted in an increase from 46% to 67% or 27% to 34% of cells in the $G_{0/1}$ phase, respectively, and a decrease from 50% to 29% or 63% to 59% of cells in the $G_{2/S}$ phase of cell cycle, respectively.

Induction of AP-1 activation by LHRH agonist triptorelin in ovarian cancer cells

EFO-21 and EFO-27 ovarian cancer cells were transiently transfected with a reporter vector containing an AP-1 enhancer fused to HSV-TK promotor and the SEAP reporter gene or the pTK-SEAP vector as a negative control. SEAP activity was detected using a chemiluminescence assay. During culture of the transfected EFO-21 or EFO-27 cells under serum and phenol red free conditions, LHRH agonist triptorelin (100 nM) treatment resulted in a 46.7 (Fig. 2A) or 44.2-fold (Fig. 2B) increase of AP-1-induced SEAP expression, respectively ($p < 0.001$).

Induction of JunD–DNA binding by LHRH agonist triptorelin in ovarian cancer cells

Activation of JunD by LHRH was analyzed using EMSA. Maximal binding of JunD on DNA consensus sequence was found after 4 h of treatment of quiescent EFO-21 (Fig. 3) or EFO-27 (not shown) human ovarian cancer cells with 100 nM LHRH agonist triptorelin.

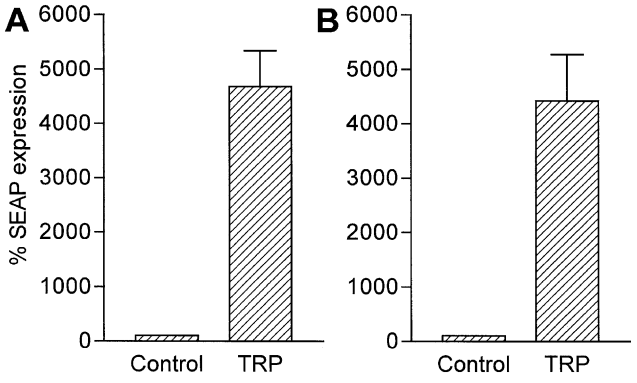

Fig. 2. AP-1 induced SEAP expression of EFO-21 (A) and EFO-27 (B) ovarian cancer cells transfected with pAP-1-SEAP plasmid with or without LHRH agonist triptorelin (100 nM) treatment. Cells were cultured for 96 h in the absence of FCS and phenol red. After treatment with triptorelin, a significant increase of SEAP expression is observed ($p < 0.001$). pAP-1-SEAP without triptorelin treatment (control); pAP-1-SEAP with triptorelin treatment (TRP). Columns represent means ± SEM of data obtained from three independent experiments run in duplicate in three different passages of each cell line.

Effects of LHRH agonist triptorelin on DNA synthesis in ovarian cancer cells

The effects of LHRH agonist triptorelin on DNA synthesis were analyzed using a [^{3}H]thymidine incorporation assay. DNA synthesis was significantly decreased to $45.5 \pm 11.4\%$ (day $0 = \text{control} = 100\%$; $p < 0.001$) after 3 days of 1 nM LHRH agonist triptorelin treatment (Fig. 4).

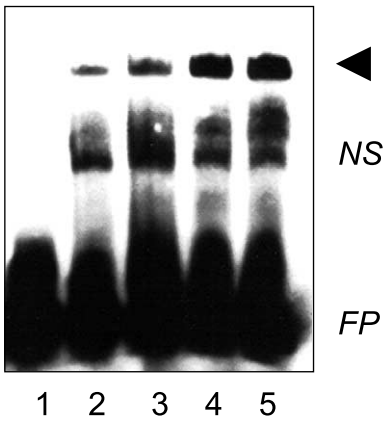

Fig. 3. EMSA analysis of nuclear protein extracts from EFO-21 ovarian cancer cells treated without (2) or with 100 nM LHRH agonist triptorelin for 2 h (3), 4 h (4), or 24 h (5) shows a time-dependent induction of JunD-binding activity (arrowhead = JunD–DNA complex). Control without nuclear proteins (1). The EMSA is representative of three separate experiments. NS, nonspecific banding; FP, free probe. Experiments using ovarian cancer cell line EFO-27 gave identical results.

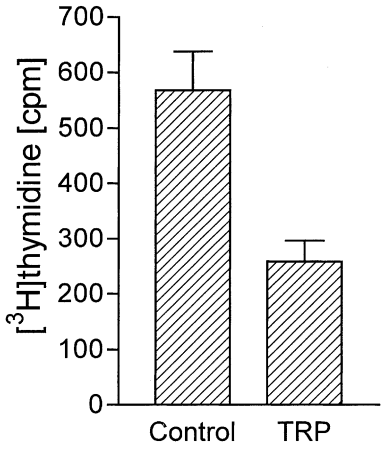

Fig. 4. Effects of 72 h treatment with 1 nM LHRH agonist triptorelin (TRP) on DNA synthesis of EFO-21 ovarian cancer cells analyzed by [^{3}H]thymidine incorporation. Columns represent means ± SEM of data obtained from six independent experiments.

Discussion

The in vitro proliferation of a variety of human cancer cell lines including cancers of the ovary can be inhibited by agonistic and/or antagonistic analogs of LHRH in a dose- and time-dependent manner (for review see [1]). This study was conducted to investigate whether regulation of cell cycle is involved in LHRH-induced downregulation of cell proliferation in primary human ovarian cancer cells derived from a woman with ovarian cancer and in two ovarian cancer cell lines. In addition, we have investigated whether the JunD/Ap-1 pathway is involved in the regulation of cell cycle by LHRH and whether LHRH affects DNA synthesis.

Treatment of primary human ovarian cancer cells with 100 nM LHRH agonist triptorelin resulted in an increase from 68% to 79% of cells in the $G_{0/1}$ phase and a decrease from 31% to 20% of cells in the $G_{2/S}$ phase of cell cycle. These data were confirmed using the ovarian cancer cell lines EFO-21 and EFO-27. Recently, Kim et al. [16] found that in ovarian cancers LHRH agonist triptorelin acts mainly by arresting cells in the $G_{0/1}$ phase of cell cycle and, thus, reduces the number of cells in S and G_2/M phases. Thompson et al. [17] also found that LHRH agonist Leuprorelin caused a reversible increase in cells in the $G_{0/1}$ phase with a corresponding decrease of cells in S and M phases. After treatment with 10 μM LHRH agonist triptorelin for 36 h, the endometrial cancer cell line CUME-1 showed a cell cycle arrest at the G_1–S transition [18]. These data together with our results suggest that the mechanism by which LHRH inhibits the growth of gynecological cancer cells may include effects on the cell cycle.

Activator protein-1 (AP-1) is activated in response to many growth factors, hormones, and other agents and it controls cell growth and programmed cell death [19]. In this study, we show that LHRH agonist triptorelin is a potent stimulator of JunD and AP-1 activity in human ovarian cancer cells. LHRH agonist triptorelin treatment (100 nM) resulted in a 46.7 or 44.2-fold increase of AP-1 activation in quiescent human ovarian cancer cells. Maximal binding of JunD on DNA consensus sequence was found after 4 h of treatment of human ovarian cancer cells with 100 nM LHRH agonist triptorelin.

Since LHRH agonist triptorelin activates the JunD/AP-1 pathway and JunD was found to be involved in an arrest of cell cycle in different cell systems [7–10], it seems to be reasonable to speculate that the JunD/AP-1 pathway is involved in the anti-proliferative actions of the LHRH agonist triptorelin in ovarian cancer cells. Overexpression of JunD in immortalized NIH 3T3 fibroblasts caused slower proliferation and accumulation of cells in $G_{0/1}$ phase, suggesting that JunD acts as a negative regulator of cell growth [7]. In the human chronic myelogenous leukemia cell line RWLeu-4 1,25-dihydroxyvitamin D3 inhibits proliferation [8]. In addition, JunD–DNA-binding activity was enhanced by 1,25-dihydroxyvitamin D3 during cell cycle arrest. In the resistant cell variant JMRD3 1,25-dihydroxyvitamin D3 had no effects on cell proliferation and JunD–DNA binding was decreased [8].

Recently, we found that LHRH agonist triptorelin induces activation of NFκB and, thus, reduces doxorubicin-induced apoptosis in human ovarian [5]. Rahmani et al. found a functional cooperation between JunD and NFκB during liver regeneration. In UT-OC-3 ovarian cancer cells, cytokines have inhibitory effects on cell proliferation and activate AP-1 and NFκB [20].

Finally, we analyzed the effects of LHRH agonist triptorelin on DNA synthesis in ovarian cancer cells

using a [^{3}H]thymidine incorporation assay. After 3 days of triptorelin treatment, DNA synthesis was decreased significantly indicating inhibition of cell proliferation and cell cycle progression.

In conclusion, our results suggest that LHRH agonist triptorelin induces JunD–DNA binding resulting in slow proliferation as indicated by increased $G_{0/1}$ phase of cell cycle and decreased DNA synthesis. In earlier investigations, we have shown that LHRH agonist triptorelin inhibits growth factor-induced mitogen-activated protein kinase (MAPK, ERK) activity [4]. In addition, LHRH agonist triptorelin downregulates growth factor-induced activation of *c-fos* expression and inhibits mitogenic signal transduction pathway [21]. Since LHRH activates NFκB and protects ovarian cancer cells from doxorubicin-induced apoptosis and JunD is shown to decrease cell cycle and cell proliferation, we propose that JunD activated by LHRH acts as a modulator of cell proliferation and cooperates with the anti-apoptotic functions of LHRH.

Acknowledgments

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