

Cellular Mechanisms of Growth Inhibition of Human Epithelial Ovarian Cancer Cell Line by LH-Releasing Hormone Antagonist Cetrorelix

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We investigated the direct effects of LH-releasing hormone (LH-RH) antagonist, Cetrorelix, on the growth of HTOA human epithelial ovarian cancer cell line. RT-PCR revealed the expression of mRNA for LH-RH and its receptor in HTOA cells. Cetrorelix, at concentrations between 10^{-9} and 10^{-5} M, exerted a dose-dependent antiproliferative action on HTOA cells, as measured by 5-bromo-2'-deoxyuridine incorporation into DNA. Flow cytometric analysis indicated that Cetrorelix, at 10^{-5} M, arrested cell cycle in HTOA cells, at G1 phase, after 24 h of treatment. Western blot analysis of cell cycle-regulatory proteins demonstrated that treatment with Cetrorelix (10^{-5} M) for 24 h did not change the steady-state levels of cyclin D1, cyclin E, and cyclin-dependent kinase (Cdk)4 but decreased the levels of cyclin A and Cdk2. The protein levels of p21 (a Cdk

inhibitor) and p53 (a suppressor of tumor cell growth and a positive regulator for p21 expression) were increased by Cetrorelix, but the levels of p27 (a Cdk inhibitor) did not change significantly. Flow cytometric analysis and terminal deoxynucleotidyltransferase-mediated deoxyuridine 5-triphosphate nick end labeling staining demonstrated that Cetrorelix (10^{-5} M) induced apoptosis in HTOA cells. In conclusion, Cetrorelix directly inhibits the proliferation of human epithelial ovarian cancer cells through mechanisms mediated by LH-RH receptor and involving multiple events in cell cycle progression, including G1 phase cell cycle arrest coupled with down-regulation of cyclin A-Cdk2 complex levels, presumably attributable to an up-regulation of p53 and p21 protein levels and apoptosis. (*J Clin Endocrinol Metab* 87: 3721–3727, 2002)

IN THE 30 yr since the elucidation of the structure of hypothalamic LH-releasing hormone (LH-RH) and its synthesis, various applications in gynecology, reproductive medicine, and oncology have been established for LH-RH agonists and antagonists (1). Various studies carried out over the past decade suggest that LH-RH analogs exert antineoplastic effects on gynecological cancer cells (1–13). Although the antiproliferative effects may be mediated by an indirect mechanism, *i.e.* a reduction in sex hormone secretion, various observations *in vitro* indicate that LH-RH analogs can directly suppress the growth of cancer cells (1–13). The expression of LH-RH and its receptor has been demonstrated in a number of human malignant tumors, including cancers of the breast, ovary, endometrium, and prostate (1, 3, 5, 7, 10, 12–17), suggesting the presence of an autocrine regulatory system of LH-RH.

Various studies revealed that LH-RH analogs act on tumor cells by interfering with the tyrosine kinase activity and the activation of mitogenic signal transduction pathway of epidermal growth factor receptor (8, 9, 18–21), a mechanism different from that operating in the pituitary, where LH-RH acts through the activation of LH-RH receptor signal transduction pathways, such as phospholipase C, protein kinase

C, and adenylyl cyclase (8). Recently, it was reported that MAPK/ERK activation may play an important role in the antiproliferative effect of LH-RH agonists (10). There are also some reports suggesting that LH-RH agonist-induced antiproliferative activity might be associated with the block of cell cycle progression in G0/G1 phase (2, 11). Some studies indicate that LH-RH antagonists induce apoptosis in cancer cells (4, 17). On the other hand, Grundker *et al.* (22) reported that LH-RH agonists protect ovarian cancer cells against chemotherapy-induced apoptosis.

LH-RH analogs have been used for the therapy of some hormone-dependent cancers, including breast, prostatic, endometrial, and ovarian cancers. However, the efficacy of LH-RH agonists in the treatment of epithelial ovarian cancer has not been conclusively demonstrated (1). According to current thinking, LH-RH antagonists may have various advantages over the agonists in the treatment of malignant tumors (1). Various antineoplastic effects of LH-RH antagonist Cetrorelix *in vivo* and *in vitro* have been reported (1, 3–7, 12, 13, 17). Our previous studies indicated that Cetrorelix inhibits the growth of OV-1063 human epithelial ovarian cancer cells in nude mice more markedly than LH-RH agonist triptorelin and may, therefore, be more efficacious clinically (1, 6). Recently, Emons *et al.* (23) reported promising effects of Cetrorelix in patients with ovarian cancer.

The exact cellular mechanisms by which LH-RH analogs suppress the growth of cancer cells are incompletely understood. Moreover, there are fewer data on LH-RH antagonists

Abbreviations: BrdU, 5-Bromo-2'-deoxyuridine; Cdk, cyclin-dependent kinase; dUTP, deoxyuridine 5-triphosphate; LH-RH, LH-releasing hormone; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

than on the agonists. In the present study, we investigated the direct effect of LH-RH antagonist Cetrorelix on the growth of HTOA human epithelial ovarian cancer cell line. Furthermore, to elucidate the cellular mechanisms by which Cetrorelix inhibits cancer cell growth, we examined the effects of Cetrorelix on cell cycle progression, the expression of cell cycle-related molecules, and apoptosis in HTOA cells.

Materials and Methods

Chemicals

LH-RH antagonist Cetrorelix [Ac-D-Nal (2)¹, D-Phe (4Cl)², D-Pal (3)³, D-Cit⁶, D-Ala¹⁰] LH-RH was originally synthesized in the laboratory of one of us (A.V.S.) (1). Some batches of Cetrorelix were kindly supplied by Zentaris (Frankfurt/Main, Germany).

DMEM-F12 medium and fetal bovine serum were purchased from Life Technologies, Inc. (Rockville, MD). All other chemicals, unless otherwise mentioned, were obtained from Sigma (St. Louis, MO).

Cell line and cell culture

Human epithelial ovarian cancer cell line HTOA was purchased from RIKEN Cell Bank of Japan (Wako, Japan). The cell line HTOA was originally established from a well-differentiated human serous cystadenocarcinoma of the ovary. HTOA cells are positive for CA 125, a marker antigen of ovarian cancer, both *in vitro* and *in vivo* (24). The cell line was grown in DMEM-F12 medium supplemented with 10% fetal bovine serum, 15 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Amphotericin B in a humidified atmosphere of 5% CO₂-95% air at 37 C.

RT-PCR

RNA was isolated from cultured HTOA cells by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippongene, Toyama, Japan). First-strand cDNA was synthesized in a reaction vol of 15 µl containing 5 µg total RNA and 0.2 µg random hexamer primers, by using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. For the detection of mRNA of LH-RH and LH-RH receptor, PCR amplification was performed with the following oligonucleotide primers: LH-RH, 5'-CTACTGACTTGGTGCCTGGA-3' (sense) and 5'-CTGCCAGTTTCTCTCAA-3' (antisense) (25); LH-RH receptor, 5'-GACCTGTCTGGAAAGATCC-3' (sense) and 5'-CAGGCTGATCAC-CACCATCA-3' (antisense) (14, 26). PCR was carried out with the Takara Taq (Takara Shuzo Co., Ltd., Otsu, Japan) in a DNA thermal cycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, CA). After an initial denaturation at 94 C for 2 min, the samples were submitted to 30 reaction cycles under the following conditions: 94 C for 30 sec, 60 C for 30 sec, and 72 C for 90 sec. PCR amplified products were electrophoresed on a 2% agarose gel containing 0.5 µg/liter ethidium bromide and photographed under UV irradiation. The PCR products were characterized by using a DNA sequencer (ABI PRISM 310 Genetic Analyzer; PE Applied Biosystems).

5-Bromo-2'-deoxyuridine (BrdU) incorporation

The effect of LH-RH antagonist Cetrorelix on the proliferation of HTOA cells was examined by measuring incorporation of BrdU into DNA by using the Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Briefly, HTOA cells were seeded into Falcon 96-multiwell plates (Becton Dickinson and Co., Franklin Lakes, NJ), at a density of 2500 cells per well, in 100 µl of the culture medium. After 24 h (d 0) and 72 h (d 2), the medium was replaced with fresh medium containing various concentrations of Cetrorelix. Cetrorelix was dissolved in dimethyl sulfoxide and diluted with the medium to yield desired concentrations. The final concentration of dimethyl sulfoxide never exceeded 0.05%. On d 1, 2, 3, and 4 of culture, 10 µl BrdU solution was added and incubated at 37 C for an additional 4 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200 µl/well fixative.

The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

Flow cytometry

HTOA cells were seeded into a 100-mm culture dish (Asahi Techno Glass, Chiba, Japan) at a density of 1×10^6 cells/dish in 10 ml of the culture medium. After 24 h, the medium was replaced with fresh medium containing 10^{-5} M Cetrorelix, and cell culture was continued for a further 8 h, 16 h, 24 h, or 32 h. Then, the cells were harvested with trypsin (0.05%)/EDTA (0.02%), washed twice with ice-cold PBS (pH 7.4), and fixed with 70% ethanol at -20 C overnight. After washing twice with ice-cold PBS, the cells were incubated in 0.25 mg/ml ribonuclease solution for 30 min at 37 C, followed by staining with 50 µl/ml propidium iodide for 30 min, on ice, in the dark. A total of 20,000 stained cells per treatment were analyzed in the EPICS XL Flow Cytometry (Beckman Coulter, Inc., Fullerton, CA).

Western blotting

HTOA cells were seeded into a 100-mm culture dish (Asahi Techno Glass) at a density of 1×10^6 cells/dish in 10 ml of the culture medium. After 24 h, the medium was replaced with fresh medium containing 10^{-5} M Cetrorelix. After an additional 24 h incubation, the cells were harvested with trypsin (0.05%)/EDTA (0.02%) and lysed in the lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 0.5% sodium deoxycholate for 30 min on ice. Insoluble material was removed by centrifugation at $12,000 \times g$, for 20 min, at 4 C. The protein (30 µg) was separated on SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). After blocking nonspecific binding sites, by incubation for 1 h with Tris buffered saline (25 mM Tris and 150 mM NaCl, pH 7.6) containing 0.2% Tween 20 and 5% nonfat milk, the membranes were blotted with the primary antibodies. Rabbit polyclonal antibodies of cyclin E, p21, p27, and p53; mouse monoclonal antibody of cyclin A; and mouse polyclonal antibodies of cyclin D1 and actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies of cyclin-dependent kinase (Cdk)2 and Cdk4 were obtained from Transduction Laboratories, Inc. (Lexington, KY). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and developed with the ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech). The images were scanned and analyzed by the fluorescence scanning system STORM (Molecular Dynamics, Inc., Sunnyvale, CA). Signals were quantitated by densitometric analysis and then expressed as a percentage of the control value.

Terminal deoxynucleotidyltransferase-mediated deoxyuridine 5-triphosphate (dUTP) nick end labeling (TUNEL)

To analyze DNA fragmentation histologically, TUNEL was performed by using the Apop Tag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, HTOA cells were seeded into Lab-Tek 8-well chamber glass slides (Nalge Nunc International, Naperville, IL), at a density of 2000 cells/chamber, in 350 µl of the culture medium. After 24 h, the medium was replaced with fresh medium containing 10^{-5} M Cetrorelix. After an additional 48 h incubation, the cells were fixed with 1% paraformaldehyde in PBS for 30 min at room temperature. After rinsing in PBS and incubation in the equilibration buffer, the slides were reacted with the TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and digoxigenin-dUTP, for 60 min at 37 C, in a humidified chamber. After washing in PBS, the labeled nucleotides incorporated into the damaged sites of DNA were marked by the antidigoxigenin peroxidase conjugate and diaminobenzidine substrate. Cells were considered positive when brown reactivity was detected in the nuclei. After counterstaining with 0.5% methyl green, the slides were analyzed under a light microscope.

Statistical analysis

Data are expressed as the mean \pm SEM from three or four independent experiments. The statistical analysis was performed by Mann-Whitney *U* test. *P* value less than 0.05 was considered statistically significant.

Results

Expression of mRNA for LH-RH receptor and LH-RH in HTOA cells

RT-PCR was performed to detect mRNA expression for LH-RH receptor and LH-RH in the human epithelial ovarian cancer cell line HTOA. As shown in Fig. 1, an amplified product with the predicted size of 319 bp for LH-RH receptor and a product with the predicted size of 240 bp for LH-RH were observed. Each PCR product was sequenced and confirmed to be identical to the sequence of LH-RH receptor or LH-RH, as previously described (25, 26).

Effect of LH-RH antagonist on DNA synthesis in HTOA cells

The effect of LH-RH antagonist Cetrorelix on DNA synthesis was examined in cultured HTOA cells. Cetrorelix, at concentrations between 10^{-9} and 10^{-5} M, produced a dose-dependent inhibition of BrdU incorporation into DNA at 48 h of treatment, with the maximal effect (55% decrease below the control) being observed at 10^{-5} M (Fig. 2A). DNA synthesis was significantly inhibited to 57% of that of the control after 24 h of incubation with Cetrorelix (10^{-5} M), and the antiproliferative effect was sustained up to 96 h (Fig. 2B).

Effect of LH-RH antagonist on cell cycle in HTOA cells

The effect of 10^{-5} M Cetrorelix on the cell cycle in HTOA cells was determined by flow cytometric analysis. As shown in Table 1, at 24 h, the proportions of HTOA cells cultured with Cetrorelix in G0/G1, S, and G2/M phases were 66.9 ± 2.8 , 22.7 ± 1.0 , and $10.7 \pm 0.7\%$, and those of the control were

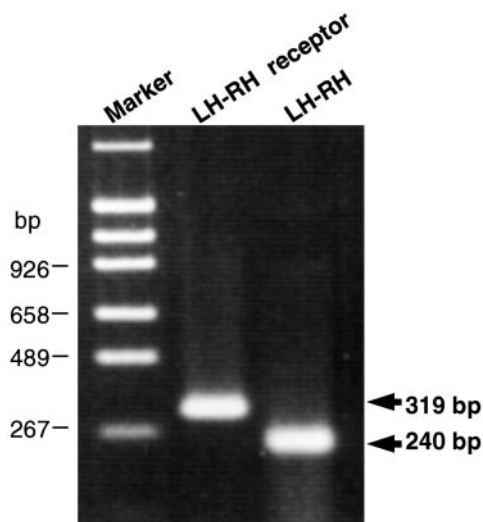


FIG. 1. Expression of LH-RH receptor and LH-RH mRNA in HTOA human epithelial ovarian cancer cells. RNA from HTOA cells was amplified with RT-PCR. Sizes of PCR products, predicted on the basis of cDNA sequence, were 319 bp for LH-RH receptor and 240 bp for LH-RH. The result is representative of three independent experiments.

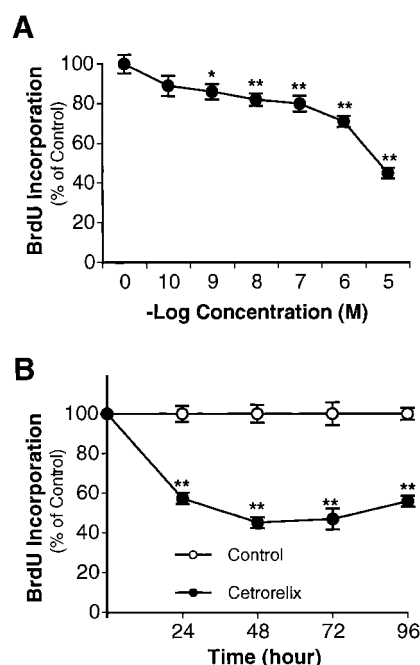


FIG. 2. Effect of LH-RH antagonist Cetrorelix on the proliferation of HTOA human epithelial ovarian cancer cells. The effect was examined by BrdU incorporation. A, Treatment with Cetrorelix at concentrations between 10^{-10} and 10^{-5} M for 48 h. B, Treatment with Cetrorelix 10^{-5} M for 24–96 h. Results are shown as the mean percentage of the untreated control \pm SEM (bars) of eight wells of three independent experiments. *, *P* < 0.05; **, *P* < 0.01 (both vs. control).

TABLE 1. Effects of LH-RH antagonist Cetrorelix on the percentage of HTOA cells in the respective phases of the cell cycle

	G0/G1 (%)	S (%)	G2/M (%)
0 h	71.7 \pm 1.3	19.8 \pm 1.1	8.5 \pm 0.3
8 h			
Control	70.4 \pm 3.2	14.8 \pm 1.1	14.7 \pm 0.6
Cetrorelix	71.0 \pm 2.2	14.5 \pm 0.9	14.5 \pm 0.2
16 h			
Control	68.8 \pm 3.5	20.2 \pm 1.0	11.0 \pm 0.9
Cetrorelix	70.4 \pm 3.2	18.2 \pm 1.1	11.4 \pm 0.8
24 h			
Control	61.6 \pm 2.7	29.1 \pm 1.3	9.2 \pm 0.2
Cetrorelix	66.9 \pm 2.8 ^a	22.7 \pm 1.0 ^a	10.7 \pm 0.7
32 h			
Control	62.3 \pm 2.8	23.2 \pm 1.2	14.5 \pm 0.5
Cetrorelix	68.8 \pm 2.2 ^a	17.8 \pm 1.0 ^a	13.4 \pm 0.2

Values are the mean \pm SEM.

^a *P* < 0.05 vs. control.

61.6 ± 2.7 , 29.1 ± 1.3 , and $9.2 \pm 0.2\%$, respectively. At 32 h, the proportions of HTOA cells cultured with Cetrorelix in G0/G1, S, and G2/M phases were 68.8 ± 2.2 , 17.8 ± 1.0 , and $13.4 \pm 0.2\%$, and those of the control were 62.3 ± 2.8 , 23.2 ± 1.2 , and $14.5 \pm 0.5\%$, respectively. The proportion of cells in G0/G1 phase was increased by 5.3% at 24 h and 6.5% at 32 h (*P* < 0.05), and that in S phase was decreased by 6.4% at 24 h and 5.4% at 32 h (*P* < 0.05) in the presence of Cetrorelix, indicating that Cetrorelix induced a significant G1 phase arrest and a concomitant reduction in the cell fraction in S phase. No significant changes in the cell cycle were observed after 8 and 16 h of incubation with Cetrorelix.

Effect of LH-RH antagonist on the expression of cell cycle-regulatory proteins in HTOA cells

We examined the protein levels of cell cycle-regulatory proteins, including cyclin A, cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, and p53 in HTOA cells exposed to Cetrorelix (10^{-5} M) for 24 h, by using Western blot analysis. Cyclin A levels were decreased by 75%, compared with the untreated control, although there were no detectable changes in the levels of cyclin D1 and cyclin E (Fig. 3A). The expression levels of Cdk2 was decreased by 22%, compared with the untreated control, but there was no significant change in Cdk4 levels (Fig. 3B). A 60% increase in p21 levels and a 50% increase in p53 levels were observed, whereas p27 levels were not changed significantly (Fig. 3C).

Induction of apoptosis by LH-RH antagonist in HTOA cells

The effect of Cetrorelix on the incidence of apoptotic cells was determined by flow cytometry and TUNEL staining in cultured HTOA cells. As shown in Fig. 4, flow cytometric analysis revealed that the proportion of sub-G1 phase, an apoptotic cell fraction, was significantly increased by the addition of 10^{-5} M Cetrorelix ($8.2 \pm 0.7\%$; $P < 0.05$), compared with that for the control ($4.2 \pm 0.5\%$) at 24 h of treatment. At 48 h, the proportion of sub-G1 phase was further increased to $9.2 \pm 0.7\%$ ($P < 0.05$), compared with the control value of $4.6 \pm 0.9\%$. The TUNEL method demonstrated that the percentage of cell death was significantly increased after 48 h of incubation with Cetrorelix ($5.0 \pm 0.4\%$; $P < 0.05$),

compared with that in the control ($1.8 \pm 0.3\%$; $P < 0.05$) (Fig. 5).

Discussion

It has been documented that LH-RH acts as an autocrine/paracrine regulator of ovarian cancer cell proliferation (27).

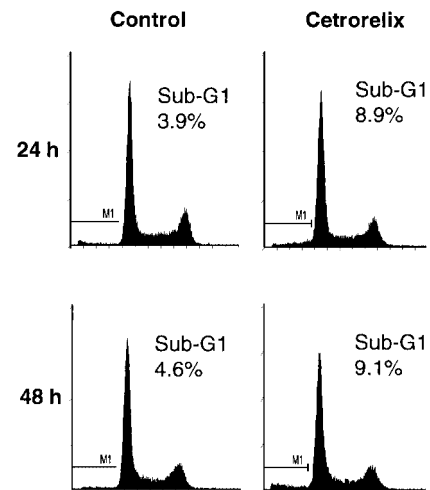


FIG. 4. Induction of apoptosis by LH-RH antagonist Cetrorelix in HTOA human epithelial ovarian cancer cells. Cells were incubated, with or without 10^{-5} M Cetrorelix, for 24 and 48 h and then analyzed by flow cytometry. Results are representative of four independent experiments. M1, Marker of subG1 phase, an apoptotic cell fraction.

FIG. 3. Effect of LH-RH antagonist Cetrorelix on the expression of cell cycle-regulatory proteins in HTOA human epithelial ovarian cancer cells. Cells were incubated, with or without 10^{-5} M Cetrorelix, for 24 h. Protein extracts were subjected to SDS-PAGE followed by Western blot analysis of protein levels of (A) cyclin A, cyclin D1, and cyclin E; (B) Cdk2 and Cdk4; and (C) p21, p27, and p53. Results on the left are representative of four independent experiments. Results on the right show quantitative analysis of each protein level (□, control; ■, Cetrorelix) and are expressed as the mean percentage of the untreated control \pm SEM (bars) of four independent experiments. *, $P < 0.05$ vs. control.

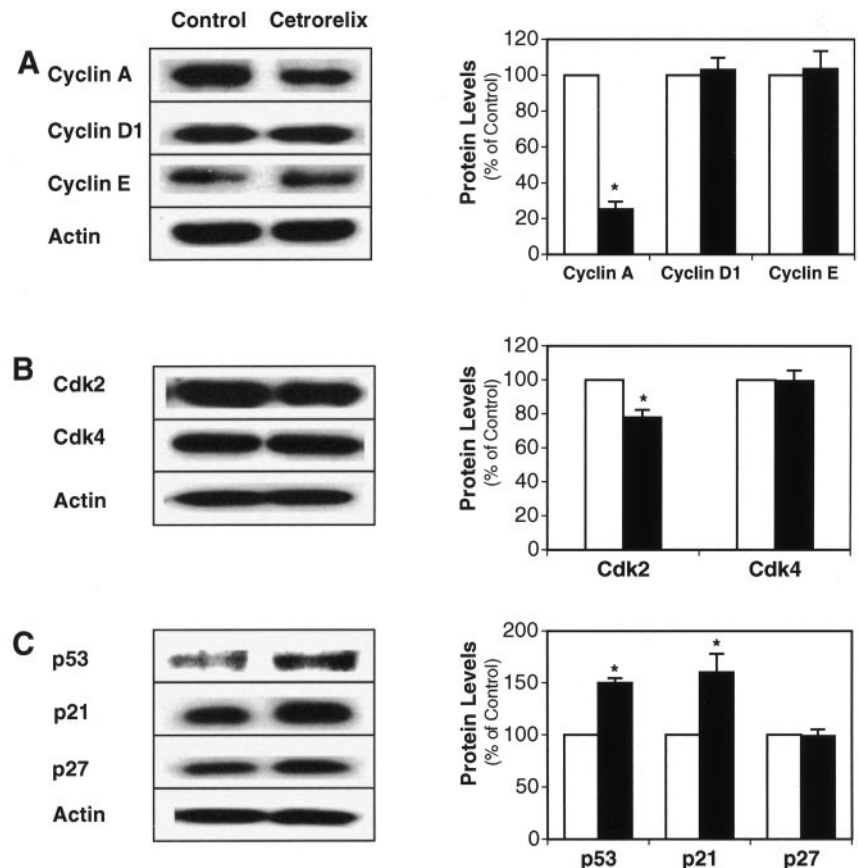
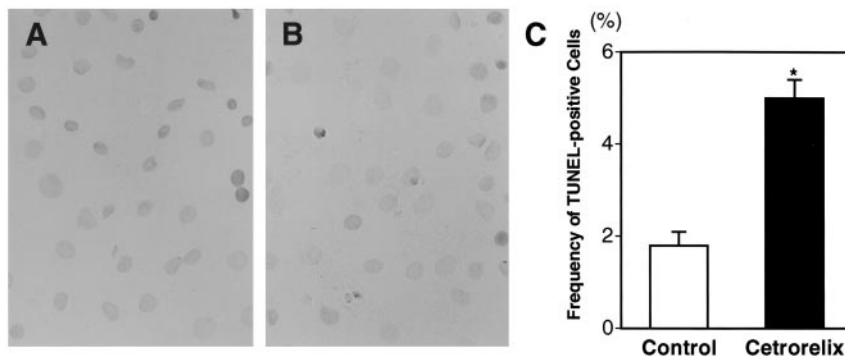


FIG. 5. TUNEL staining of LH-RH antagonist-treated HTOA human epithelial ovarian cancer cells. TUNEL-positive nuclei are stained brown. A, Control ($\times 200$). B, Cetrorelix (10^{-5} M) increased the rate of TUNEL-positive cells at 48 h of the treatment ($\times 200$). C, Frequency of TUNEL-positive cells, which was calculated by counting the number of stained cells per more than 200 cells. Results are shown as the mean \pm SEM. *, $P < 0.05$ vs. control.



In the present study, using RT-PCR, we demonstrated that mRNA for LH-RH and its receptor is also expressed in HTOA human epithelial ovarian cancer cells, suggesting that an autocrine/paracrine LH-RH loop may be present in HTOA cells and may participate in the regulation of cell growth.

Current studies have demonstrated that Cetrorelix inhibits the growth of various experimental tumors and suppresses the proliferation of prostatic, breast, ovarian, and endometrial cancer cells directly through a mechanism mediated by specific LH-RH receptors (1, 3–7, 13, 17). The present study showed that Cetrorelix exerts a dose-dependent antiproliferative action in HTOA cells, as determined by BrdU incorporation into DNA, indicating that Cetrorelix possesses a direct inhibitory effect on the proliferation of these cells.

Cell cycle analysis revealed that Cetrorelix (10^{-5} M) was able to induce G1 phase arrest of HTOA cells at 24 h of treatment, accompanied by a decrease in the cells in S phase. To gain an insight into these changes, we further examined the effect of Cetrorelix on regulatory proteins of the cell cycle. The progression of cell cycle is governed by specific activation and subsequent inactivation of Cdks, whose activity is regulated by cyclin binding, phosphorylation, and associated inhibitory molecules (28–30). In particular, cell cycle progression through G1 to S phase is regulated by the activities of D-type cyclin and A- or E-type cyclin, in association with Cdk4/Cdk6 and Cdk2, respectively (30). The cyclin D1-Cdk4/Cdk6 complexes and cyclin E-Cdk2 complex are activated in G1 phase. Human cyclin A was shown to bind and activate Cdk2 in S and G2 phases (31, 32). There is evidence suggesting that in somatic mammalian cells, cyclin A also has a primary role in the regulation of G1-S transition. It was similarly demonstrated that deregulated expression of cyclin A perturbs the normal regulation of the G1-to-S transition (33). The onset of cyclin A synthesis in late G1 is important for the G1-S transition, because the inhibition of cyclin A function in cells also inhibits the entry into S phase (31, 34). In the present study, we detected a significant decrease in cyclin A level but no changes in the protein levels of cyclin D1 and cyclin E. In addition, there was a decreased protein level of Cdk2, whereas the protein level of Cdk4 was stable. Thus, it seems that Cetrorelix may inhibit the growth of HTOA cells by acting on the cells in late G1 phase, possibly through a mechanism involving a reduction in the expression of cyclin A and Cdk2. At present, some findings on the effect of LH-RH agonist on cell cycle are available. For instance,

LH-RH agonist was shown to block cell cycle progression in G0/G1 phase, coupled with a decrease in the cells in S and G2/M phases (2, 11). In addition, LH-RH agonist decreases the expression of Cdk1, possibly as a result of the blocking of G0/G1 phase (11). However, to our knowledge, there are presently no reports on the modulation of cell cycle and cell cycle regulatory factors by LH-RH antagonists.

The activity of cyclin Cdk complexes is negatively regulated by Cdk inhibitors such as p21 and p27 (29, 33, 35). In our study, G1 arrest in HTOA cells was associated with a marked up-regulation of p21, which acts ubiquitously on Cdks and is responsible for the induction of cell cycle arrest (36), whereas there was no detectable change in p27 protein level. It is known that p21 can be up-regulated by both p53-dependent and p53-independent pathways (37–39). The biological functions of wild-type p53 are mainly related to: 1) cell-cycle arrest/growth arrest; 2) induction of apoptosis; and 3) maintenance of genetic stability by modulation of DNA repair, replication, and recombination machinery (40). Enhanced expression of p53 gives rise to G1 arrest alone, G2/M arrest, or both G1 and G2/M arrests, depending on the cell type (41–43). In this study, the expression of p53 protein was enhanced in association with the up-regulation of p21 protein. Thus, it is possible that p21 induction and the cell cycle arrest of G1 phase in HTOA cells may be mediated by p53.

Several studies showed that the antiproliferative activity induced by antagonists and agonists of LH-RH might be mediated by stimulation of apoptotic cell death (4, 17, 44, 45). On the other hand, it was reported that LH-RH agonist induced the activation of nuclear factor κ B and inhibited chemotherapy-induced apoptosis in ovarian cancer cells (22). In the present study, flow cytometry and TUNEL staining revealed the induction of apoptosis in the presence of Cetrorelix in HTOA cells, indicating that apoptosis may be one way of explaining the mechanisms of the antiproliferative effect of Cetrorelix on HTOA cells. Recent studies demonstrate that LH-RH agonists induce Fas-ligand production in LH-RH receptor-positive ovarian and endometrial cancer cells and inhibit the growth of Fas-positive endometrial cancer cells (44, 45). Although we found the expression of Fas mRNA in HTOA cells, by RT-PCR, Fas-ligand expression was not induced by Cetrorelix (data not shown). This suggests that, in our experimental model, the apoptosis induced by LH-RH antagonist Cetrorelix in HTOA cells may not be mediated through Fas/Fas-ligand pathway. However, it is

possible that the up-regulation of p53 and p21 also mediates apoptosis in Cetrorelix-treated HTOA cells (40, 46). The molecular mechanisms by which Cetrorelix induced apoptosis in HTOA cells need to be further investigated.

In summary, we demonstrated that LH-RH antagonist Cetrorelix inhibits directly the growth of HTOA human epithelial ovarian cancer cells that express LH-RH and LH-RH receptor mRNA. This inhibitory effect might be, at least in part, attributed to G1 phase arrest, the likely mechanisms involving the down-regulation of Cdk2 and cyclin A protein levels and the up-regulation of p21 and p53 protein levels, as well as apoptosis.

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