Abstract. Luteinizing hormone-releasing hormone (LHRH) and its receptor are frequently expressed in human ovarian and endometrial cancers and are part of an autocrine mechanism of growth control. We have previously shown that the LHRH analog Triptorelin induces activation of nucleus factor kappa B (NFκB) and reduces apoptosis induced by doxorubicin in human ovarian cancer cells EFO-21 and EFO-27. The present study was performed to investigate the anti-apoptotic effects of LHRH analogs on apoptosis induced by doxorubicin, UV-light and ligation of CD95 in human endometrial and ovarian cancer cells. We further investigated the interaction of the LHRH system with the apoptotic pathway focusing on the effector-protease caspase 3. Doxorubicin (100 nM) induced apoptosis in the LHRH-receptor-positive human endometrial cancer cell line Ishikawa and in the human ovarian cancer cell lines EFO-21 and NIH:OVCAR-3. Pretreatment for 24 h with native LHRH, the LHRH agonist Triptorelin or the LHRH antagonist Cetrorelix (100 nM) significantly reduced apoptosis induced by doxorubicin in these cells. In EFO-21 cells pretreatment with 100 nM Triptorelin also reduced UV-light-induced apoptosis from 76% to 62.7% (p<0.01). EFO-21 cells express CD95. Cross-linking of CD95 with monoclonal antibody anti-APO-1 (500 ng/ml) increased apoptosis from spontaneous rate to 10.3% to 38.3% in EFO-21 cells (p<0.001). Pre-treatment with Triptorelin did not reduce CD95-mediated apoptosis in these cells. LHRH analogs protect human endometrial and ovarian cancer cells from DNA-replication-dependent cytotoxic agent and UV-light-induced apoptosis, but not from CD95-mediated apoptosis.

The expression of luteinizing hormone-releasing hormone (LHRH) and its receptor as a part of an autocrine regulatory system of cell proliferation has been demonstrated in a number of human malignant tumors, including cancers of the breast, ovary and endometrium (1-4). Dose-dependent antiproliferative effects of LHRH analogs in cell lines derived from these cancers have been observed by various investigators (2, 5-8). Both, LHRH agonists and antagonists have marked antiproliferative 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. The classical LHRH receptor signal-transduction mechanisms, known to operate in the pituitary, are not involved in the mediation of antiproliferative effects of LHRH analogs in cancer cells. 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 down-regulation of cancer cell proliferation (9). In addition, LHRH activates nucleus factor κB (NFκB) and protects the cancer cells from doxorubicin-induced apoptosis (10). Imai et al. reported LHRH-induced activation of the apoptotic cascade (11-13). Though we have tried to show induction of apoptosis by LHRH analogs, we have found it only in one ovarian (Ca-Ov-3) out of nine ovarian (EFO-21, EFO-27, NIH:OVCAR-3, AN-3-CA, Ca-Ov-3, SK-OV-3) and endometrial (HEC-1A, HEC-1B, Ishikawa) cell lines (10; unpublished results). Inhibition of NFκB can induce apoptosis and potentiate TNF-α- and chemotherapy-induced apoptosis (14, 15). Additionally, activation of NFκB has been shown to inhibit apoptosis by blocking caspase cleavage and cytochrome c release (16-19).

Studies with the nematode Caenorhabditis elegans have shown that specific genes are necessary for apoptosis to occur, like ced-3 and ced-4, whereas other genes such as ced-
9 protect from cell death (20, 21). All members of the ced-3 family are found as inactive zymogens that become activated by proteolytic cleavage to the active dimeric or tetrameric species (22). These cysteine proteases, formerly known as the ICE (interleukin 1β converting enzyme) family, are now called caspases and contain around 10 members. These proteases drive the effector process of apoptosis.

This study was conducted to investigate the anti-apoptotic effects of native LHRH and its analogs Triptorelin and Cetrorelix in the human endometrial cancer cell line Ishikawa and ovarian cancer cell lines EFO-21 and NIH:OVCAR-3. We first evaluated the effects of LHRH analogs on doxorubicin-induced apoptosis in these cells. Then we attempted to show that LHRH also inhibits UV-light-induced apoptosis in EFO-21 cells. Further we analysed the effects of LHRH on CD95-mediated apoptosis. Finally we attempted to investigate the interference of LHRH with apoptotic signal transduction. We focused our interest on the effector-protease caspase 3 (CPP32).

Materials and Methods

Reagents and antibodies. Triptorelin was kindly provided by Ferring-Arzneimittel Ltd. (Kiel, Germany). Cetrorelix (SB-75) was provided by Zentaris (Frankfurt, Germany). LH-RH, propidium-iodide (PI), gamma-interferon (IFN-γ) and FITC-conjugated goat anti-mouse IgG-antibody were purchased from Sigma Chemical Co (St. Louis, MO, USA). Anti-APO-1-antibody (IgG3) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). The caspase 3-activity assay PhiPhiLux-G2D2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD, USA).

Cell lines, culture conditions and induction of apoptosis. The human ovarian cancer cell lines EFO-21 and NIH:OVCAR-3 and the endometrial cancer cell line Ishikawa were cultured as described in detail previously (1-4). Cells were kept in medium containing 10% FCS (fetal calf serum, Biochrom, Berlin, Germany), 5 mM L-glutamine, 100 U/ml penicillin and 100 Ìg/ml streptomycin. Apoptosis was induced by treatment with doxorubicin (100 nM) up to 72 h, or by exposure to UV-light (254 nm, 15 W) for 30 sec. Cells were kept in culture for 72 h. CD95-positive EFO-21 cells were first tested for effects of monoclonal antibody anti-APO-1 by cross-linking CD95. At a minimum concentration of 500 ng/ml a significant increase of apoptotic cells could be observed after 24 h. Longer duration of incubation with anti-APO-1 did not further increase the apoptosis rate. To inhibit apoptosis with LHRH analogs via NFκB-activation, cells were pre-treated with LHRH analogs at 100 nM 24 h before cytotoxic agent, UV-light or anti-APO-1 treatment was performed.

Flow cytometry. To analyse CD95-expression, 10⁶ cells were resuspended in 50 µl Hank’s salt solution containing 0.1% sodium azide, 1.5% HEPES and 2% FCS. Fifty µl of diluted primary antibody (1:20) was added and incubated on ice for 1 h. After washing, FITC-conjugated goat anti-mouse IgG at appropriate dilution (1:20) was added. The cell suspension was incubated on ice for 1 h. Cells were washed in Hank’s salt solution and immediately analysed by flow cytometry on FACScalibur® equipment (Becton Dickinson Immunocytometry Sys., Mountain View, CA, USA) using Cellquest software. To quantify cells with advanced DNA degradation, we used a procedure similar to that described by Nicoletti et al. (23). A pellet containing 1 x 10⁶ cells was gently resuspended in 500 µl of hypotonic fluorochrome solution containing 0.1% Triton X-100 (Sigma Chemical Co.), 0.1% sodium citrate and 50 µg/ml PI. The cell suspensions were placed at 4°C in the dark overnight before flow cytometry analysis of cellular DNA content. Caspase 3 activity was observed with cell-permeable fluorogenic caspase substrate PhiPhiLux (24). Preparation of PhiPhiLux-G2D2 was performed in accordance with the supplier’s instructions and measured by flow cytometry on FACScalibur® equipment (Becton Dickinson).

Statistical analysis. All experiments were performed at least 3 times in different passages of the respective cell lines. 10⁵ cells were counted in each experiment. Results were tested by one-way analysis of variance, followed by a Newman Keuls’ test for the comparison of individual groups.

Results

Effects of LHRH, Triptorelin and Cetrorelix on apoptosis induced by doxorubicin. Ishikawa, EFO-21 and NIH:OVCAR-3 cells were first treated with either native LHRH, Triptorelin or Cetrorelix (100 nM). After 72 h of
treatment no increased apoptosis rate was observed (data not shown). Cells were then pre-treated for 24 h with LHRH or its analogs (100 nM) before the cytotoxic agent doxorubicin was added (100 nM). After a further treatment of 72 h with doxorubicin, the cells showed morphological changes characteristic of apoptosis and cells with advanced DNA fragmentation were quantified by flow cytometry. In human ovarian cancer cells, EFO-21 apoptosis induced by doxorubicin increased from the spontaneous rate of 10.3±2.5% to 44.3±3.2% (p<0.001). Pre-treatment with Triptorelin, Cetrorelix or native LHRH significantly reduced doxorubicin-induced apoptosis in EFO-21 cells (18.0±2.6%, 23.3±1.5% and 25.7±3.1%, respectively; all p<0.001) (Figure 1). In human endometrial carcinoma cells Ishikawa apoptosis induced by doxorubicin increased from the spontaneous rate of 21.7±2.5% to 74.3±5.7% (p<0.001). Pre-treatment with Triptorelin, Cetrorelix or native LHRH significantly reduced doxorubicin-induced apoptosis in Ishikawa cells (31.3±1.5%, 33±4.4% and 44.7±2.5%, respectively; all p<0.001) (Figure 1). Comparable results were obtained with NIH:OVCAR-3 cells (data not shown).

Effects of Triptorelin on apoptosis induced by UV-light. Seventy-two h after exposure to UV-light (30 sec) EFO-21 cells showed morphological criteria of apoptosis and cells with advanced DNA fragmentation were quantified by flow cytometry. Apoptosis induced by UV-light increased from the spontaneous rate of 10.7±1.5% to 76±3.6% (p<0.001). Pre-treatment with Triptorelin (24 h, 100 nM) reduced UV-light-induced apoptosis to 62.7±5% (p<0.01) (Figure 2).

Effects of Triptorelin on CD95-mediated apoptosis. Flow cytometry analysis revealed slight CD95 expression in EFO-21 cells. However, treatment with escalating doses of IFN-γ did not increase surface expression of CD95 in these cells (data not shown). After 24 h of treatment with anti-APO-1 (500 ng/ml), apoptosis increased from a 10.3±2.5% spontaneous apoptosis rate to 38.3±2.5% (p<0.001). Pre-treatment with Triptorelin (24 h, 100 nM) did not reduce the anti-APO-1-induced apoptosis (40±3.0%) (Figure 3).

Figure 2. Percentage of apoptotic cells measured by flow cytometry after 72 h of treatment for 30 sec with UV-light in EFO-21 cells. Triptorelin, Cetrorelix or native LHRH (all 100 nM) were added 24 h before exposure to UV-light (254 nm, 15 W). Columns represent means plus SE of data obtained from 3 independent experiments in 3 different passages of the cell lines. Results were tested by one-way analysis of variance, followed by a Newman Keuls' test for the comparison of individual groups. [a: p<0.001 versus control; b: p<0.01 versus UV]

Figure 3. Percentage of apoptotic cells measured by flow cytometry after 24 h of treatment with anti-APO-1 (500 ng/ml) in EFO-21 cells. Triptorelin, Cetrorelix or native LHRH (all 100 nM) were added 24 h before treatment with anti-APO-1. Columns represent means plus SE of data obtained from 3 independent experiments in 3 different passages of the cell lines. Results were tested by one-way analysis of variance, followed by a Newman Keuls' test for the comparison of individual groups. [a: p<0.001 versus control]
Effects of LHRH agonist on caspase 3-activity. Exposure to doxorubicin (100 nM) for 24 h increased caspase 3 activity in EFO-21 and NIH:OVCAR-3 cells as measured by the cleavage of the fluorescent CPP32-like substrate PhiPhiLux-G2D2. Pre-treatment with Triptorelin (24 h, 100 nM) had no inhibitory effects on doxorubicin-induced caspase 3 activity (Figure 4). In Ishikawa cells no increase of caspase 3 activity was detectable (Figure 4).

Discussion

The present study showed that LHRH, its agonist Triptorelin and its antagonist Cetrorelix inhibit apoptosis induced by cytotoxic agent and UV-light in LHRH-receptor-positive human endometrial and ovarian cancers. Pre-treatment with Triptorelin failed to reduce CD95-mediated apoptosis. However, in our system the activity of the effector-protease caspase 3 did not correlate with the amount of apoptosis in the endometrial cancer cells Ishikawa and ovarian cancer cells EFO-21 and EFO-27.

We previously demonstrated that Triptorelin activates NFkB and reduces doxorubicin-induced apoptosis in EFO-21 and EFO-27 cells when simultaneously treated with doxorubicin and Triptorelin (10). When cells were pretreated with Triptorelin before the cytotoxic agent was added, inhibition of apoptosis by doxorubicin was more pronounced compared to simultaneous treatment, a finding that suggests a time-dependent activation of NFkB. Activation of NFkB has been shown to interfere with the caspase-cascade and to protect cells from chemotherapy-induced apoptosis (15, 19). When cells receive signals that activate NFkB, the inhibitor I kB is phosphorylated and degraded and translocation of NFkB from the cytoplasm into the nucleus is triggered. Since Triptorelin has been shown to induce NFkB-activity and the JNK/c-jun pathway (10, 25) which activates AP-1, it is likely that LHRH-
mediated inhibition of proliferation allows for DNA-repair mechanisms and prohibits DNA-damage. Using PCR we detected mRNA for p53 in all cell lines used in our investigations (own unpublished data). Interestingly, Tergoankar et al. described inhibition of chemotherapy-induced activation of p53 and apoptosis by activation of NFκB, implying a role for NFκB in acquisition of resistance to chemotherapy (26).

Apoptosis induced by doxorubicin or by UV-light depends on ongoing DNA replication. Trimerization of CD95 by its ligand (CD95-L) or through cross-linking by the monoclonal antibody anti-APO-1 initiates a chain of protein-protein interactions via FADD (fas-activated death domain) and newly discovered ICE-family (interleukin-1β converting enzyme) proteases, FLICE 1 and 2 (FADD-homologous ICE/CED-3-like protease)(27-29). CD95-mediated apoptosis is completed much more rapidly than apoptosis induced by cytotoxic agent or UV-light. This could be explained by direct, non-transcriptional coupling between the activation of a cytokine receptor by its ligand and the effector proteases. Probably because of this non-transcriptional process, NFκB-activation by LHRH failed to inhibit CD95-mediated apoptosis in EFO-21 cells.

To investigate whether LHRH-induced inhibition of apoptosis is due to interference with the effector process of apoptosis by blocking caspase cleavage, we focused our interest on the effector caspase 3 (CPP32). Caspase 3 cleaves poly [ADP-ribose] polymerase (PARP) and reacts as an essential effector caspase (30-33). Recent studies described caspase 3-independent apoptosis-induction by TGF-β1 in Burkitt’s lymphoma cells, or in macrophages by exogenously added nitric oxide donors, implying that there are other, as yet unidentified, executioner caspasess (34, 35). Pagliari et al. described caspase 3-independent apoptosis in macrophages by selective down-regulation of NFκB and consecutive reduction of A1 mRNA levels (member of the Bcl-2 family), followed by loss of mitochondrial transmembrane potential (36). In addition, Cenni et al. reported enhancement of cisplatin-induced apoptosis by epidermal growth factor by a caspase 3-independent pathway (37). However, although caspase 3 activity is enhanced in EFO-21 and NIH:OVCAR-3 cells when treated with doxorubicin, its activity does not correlate with the final apoptosis rate and is not reduced by LHRH-mediated activation of NFκB. Additionally, caspase 3 was not induced in human endometrial carcinoma cells Ishikawa by doxorubicin. These findings indicate an apoptotic pathway independent from caspase 3 in gynecologic malignancies.

LHRH activates NFκB and protects LHRH-receptor-positive cells from apoptosis induced by cytotoxic agent and UV-light but not from apoptosis mediated through CD95. Razandi et al. recently showed anti-apoptotic effects by inhibition of Taxol or UV-stimulated c-Jun N-terminal kinase (JNK) activity by estradiol in human breast cancer cells, probably mediated through the plasma membrane receptor (38). It is likely that LHRH interferes with other signal transduction mechanisms which inhibit rapid proliferation but protect the cells from proliferation-dependent apoptosis, but not from CD95-mediated apoptosis. Further research on these mechanisms is required to open new therapeutic strategies.

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