Luteinizing hormone inhibits Fas-induced apoptosis in ovarian surface epithelial cell lines

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Abstract

Gonadotrophins including LH have been suggested to play an important role in the etiology of epithelial ovarian cancers. The goal of the present study was to obtain more insight in the mechanism of gonadotrophin action on ovarian surface epithelium (OSE) cells. As the Fas system is known to be a major player in the regulation of the process of apoptosis in the ovary, we investigated whether LH interfered with Fas-induced apoptosis in the human OSE cancer cell lines HEY and Caov-3. Activation of Fas receptor by an agonistic anti-Fas receptor antibody induced apoptosis, as was evaluated by caspase-3 activation, poly(ADP-ribose) polymerase fragmentation, phosphatidylserine externalization and morphological changes characteristic of apoptosis. Co-treatment with LH

reduced the number of apoptotic cells following activation of Fas in a transient manner, while LH by itself did not affect apoptosis or cell proliferation. The anti-apoptotic effect of LH could be mimicked by the membrane-permeable cAMP analog 8-(4-chlorophenylthio)cAMP (8-CPT-cAMP), and blocked by H89, a specific inhibitor of protein kinase A (PKA). In conclusion, these findings suggest that LH protects HEY cells against Fas-induced apoptosis through a signaling cascade involving PKA. Although it is plausible that *in vivo* LH might also enhance OSE tumor growth through inhibition of apoptosis, further research is necessary to confirm this hypothesis.

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Introduction

Although epithelial ovarian cancer is the most lethal of all gynecological cancers in women in the Western world, the etiology of this disease remains poorly understood (Auersperg *et al.* 2001). An inherited mutation in the BRCA1 gene and to a lesser extent in the BRCA2 gene has been implicated in 5–10% of all cases (Ford *et al.* 1995). Another major risk factor for the development of epithelial ovarian cancer is related to the number of lifetime ovulations (Casagrande *et al.* 1979). The subsequent repeated rupture and repair of ovarian surface epithelium (OSE) at the ovulation site requires a high proliferative activity of the epithelium, resulting in an increased risk of mutations in these cells (Fathalla 1971). Moreover, OSE cells sequestered in crypts and inclusion cysts are exposed to high levels of stroma-derived growth factors and steroids, which might also contribute to ovarian cancer development (Cramer & Welch 1983).

Evidence in support of hormonal involvement in ovarian carcinogenesis has been given by the observation that lower basal and peak gonadotrophin levels,

as occurring in women during use of oral contraceptives, pregnancy and breastfeeding, were found to protect against ovarian cancer (Cramer *et al.* 1983, Whittemore *et al.* 1992). Conversely, the incidence of OSE cancer is increased in women during the years after menopause when serum gonadotrophin levels are high (Wu *et al.* 1988). Moreover, cases have been reported of OSE cancers arising in infertile women during or after prolonged treatment with gonadotrophins (Anderson & Dimitrievich 1996, Kuroda *et al.* 1998). Considering the number of women receiving assisted reproductive treatment nowadays and the expected growing demand for ovulation induction during the coming years, it is of major importance to elucidate the role of gonadotrophins in the development and progression of epithelial ovarian cancer.

Until now, it is unclear whether gonadotrophins, like luteinizing hormone (LH), act in an indirect endocrine manner (via stimulating steroid production in ovarian follicular cells) or directly target OSE cells. Expression of LH receptors (LH-Rs) has been demonstrated in normal OSE cells (Parrott *et al.* 2001a), in epithelial cells lining ovarian inclusion cysts (Kuroda *et al.* 1998) and

in approximately 50% of ovarian carcinomas (Mandai *et al.* 1997). However, controversy exists as to whether there is a direct effect of LH on OSE cell survival, as increased (Casagrande *et al.* 1979, Simon *et al.* 1983, Osterholzer *et al.* 1985, Wimalasena *et al.* 1992, Syed *et al.* 2001, Parrott *et al.* 2001a), unchanged (Ala-Fossi *et al.* 1999, Ivarsson *et al.* 2001, Wright *et al.* 2002) as well as decreased (Zheng *et al.* 2000, Tourgeman *et al.* 2002) proliferation rates in response to LH have been reported. Many OSE tumors have been shown to contain low apoptotic indices and, therefore, it may be possible that LH influences OSE tumor growth and survival through inhibition of apoptosis. Indeed, it has been demonstrated that human chorionic gonadotrophin (hCG), an LH analog, suppressed apoptosis of ovarian granulosa cells (Chun *et al.* 1994). However, not much is known about the effects of LH on apoptosis in OSE cells.

A major pathway triggering apoptosis in OSE cells involves the Fas system (Ghahremani *et al.* 1998). Fas receptor (CD95) is a member of the tumor necrosis factor/nerve growth factor family which is expressed in many OSE cancers (Ben Hur *et al.* 1999, Das *et al.* 2000). Fas is activated by binding of Fas ligand, leading to activation of downstream death executioner factors, such as caspase-3, eventually resulting in cell death (Krammer 1999). Fas-induced apoptosis is also believed to be one of the mechanisms involved in cisplatin cytotoxicity in OSE cancer cells (Uslu *et al.* 1996, Wakahara *et al.* 1997), a therapy often used as treatment for OSE cancer. We have examined the effect of LH on the occurrence of Fas-induced apoptosis in the human ovarian epithelial cancer cell lines HEY and Caov-3 and determined whether signaling occurs via protein kinase A (PKA) and/or protein kinase C (PKC) activation.

Materials and Methods

Reagents

All cell culture reagents were purchased from Gibco BRL, except for the medium, minimum essential medium without Phenol Red (M3149), which was obtained from Sigma. H89, an inhibitor of PKA, 8-(4-chlorophenylthio)cAMP (8-CPT-cAMP), H7, an inhibitor of PKC, 4- β -phorbol-12-myristate-13-acetate (PMA), an activator of PKC, 4- α -phorbol-12-myristate-13-acetate, an inactive phorbol ester, and 3-isobutyl-1-methyl-xanthine (IBMX), a phosphodiesterase inhibitor, were also purchased from Sigma. The agonistic mouse anti-human Fas IgM (clone CH11) was obtained from Upstate Biotechnology (Campore Scientific, Veenendaal, The Netherlands), the caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) from the Promega Corporation, and ovine LH (NIH-LH-S20) was a gift from NIDH (Bethesda, MD, USA). The antibody against the LH-R (P1B4) was a gift from

Dr J Wimalasena (University of Tennessee, Knoxville, TN, USA). This antibody was raised against purified rat LH-Rs, as described by Indrapichate and colleagues (1992) and has been shown to bind specifically to LH-Rs in different tissues (Bukovsky *et al.* 1993). Antibodies against Fas receptor (sc-715), Fas ligand (sc-956) and procaspase-3 (sc-1226) were purchased from Santa Cruz Biotechnology (SanverTech, Heerhugowaard, The Netherlands). The antibodies against poly(ADP-ribose) polymerase (PARP) p85 (G7341), active caspase-3 (AF835) and caspase-3 (A3537) were obtained from the Promega Corporation, R&D Systems (ITK Diagnostics Uithoorn, The Netherlands) and DakoCytomation (Heverlee, The Netherlands), respectively. Secondary biotinylated goat anti-rabbit, rabbit anti-goat or goat anti-mouse IgGs were purchased from Vector Laboratories (Vectastain kit Elite; Burlingame, CA, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG was from Nordic Immunological Laboratories (Tilburg, The Netherlands). The bromodeoxyuridine (BrdU) incorporation assay detection kit and the annexin-V-Fluor staining kit were purchased from Roche Diagnostics, the cAMP Biotrak enzyme-immunoassay system was from Amersham Biosciences and the Supersignal chemiluminescence substrate kit (ECL) was from Pierce (Tattenhall, Cheshire, UK).

Cell culture

For this study, the human OSE cell lines HEY and Caov-3 were used. The HEY cell line was originally derived from a papillary cystadenocarcinoma (Jindal *et al.* 1995); while the Caov-3 was derived from an ovarian adenocarcinoma (American Type Culture Collection HTB-75; Manassas, VA, USA). Both cell lines were kindly provided by Dr D Lobb (McMasters University, Hamilton, Canada) and were routinely kept in culture at 37 °C in a 5% CO₂ incubator in Eagle's minimal essential medium without Phenol Red. The culture medium was supplemented with 0.1 mM non-essential amino acids, 2 mM glutamine, 50 U/ml penicillin/streptomycin, 1.5 mM Hepes and 10% heat-inactivated fetal calf serum. The cells were sub-cultured twice a week and only those cells in the exponential growth phase were used in the experiments described.

Immunohistochemistry

To detect the LH-R, Fas receptor (Fas), Fas ligand, procaspase-3, active caspase-3 and fragmented PARP in OSE cancer cell lines, cells were grown on glass coverslips placed in 24-multiwell culture dishes and seeded at a density of 2.5×10^4 cells/well. After incubation overnight the culture medium was replaced by serum-free medium and cells were cultured for another 24 h. Then the cells were fixed for 15 min in 4% buffered formalin, pH 7.4,

and immunohistochemistry was performed as been described previously (Teerds & Dorrington 1995). Briefly, cells were washed in 0.01 M Tris-buffered saline (TBS; pH 7.4), and incubated with 0.1 M glycine in TBS for 30 min. After rinsing, cells were permeabilized with 0.1% Triton X-100 in TBS for 5 min. Cells were, depending on the species in which the secondary antibody was raised, blocked for 30 min with 10% normal rabbit, goat or mouse serum in TBS, and then incubated overnight at 4 °C with rabbit anti-human polyclonal antibodies against Fas, Fas ligand (dilution 1:100), active caspase-3 (0.5 µg/ml) or PARP p85 (dilution 1:100), with a goat anti-human polyclonal antibody against procaspase-3 (dilution 1:100) or with a mouse anti-rat monoclonal antibody against LH-R (dilution 1:3000). All antibodies were diluted in TBS containing 0.05% acetylated BSA (Aurion, Wageningen, The Netherlands). After washing the cells in TBS, the cells were incubated with the corresponding biotinylated goat anti-rabbit (Fas, Fas ligand, active caspase-3 and PARP p85), rabbit anti-goat (procaspase-3) or goat anti-mouse (LH-R) antibodies diluted 1:200 in TBS containing 0.05% acetylated BSA for 60 min. Cells were again washed in TBS and subsequently incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining kit Elite. Both components (A and B) were diluted 1:1000 and prepared at least 15 min before use. Then, cells were washed in TBS, and bound antibody was visualized after the addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetra-chloride (Sigma) in TBS to which 0.03% H₂O₂ was added. Finally, the cells were counterstained with Mayer's hematoxylin. Controls, in which the primary antibody was replaced by normal rabbit, goat or mouse serum respectively, were processed similarly. No staining was observed in these controls.

Western blot analysis

Cells seeded at an initial density of 2.5×10^5 cells/dish in 20 cm² culture dishes were incubated overnight in culture medium. Next, the culture medium was replaced by serum-free culture medium supplemented with or without agonistic anti-Fas antibody (50 ng/ml CH11) and a caspase inhibitor, Z-VAD-FMK (10^{-5} M), and incubated for 4, 8 or 24 h. Culture medium was collected and centrifuged for 5 min at 6000 g to spin down the detached cells. Adherent cells were scraped from the surface following the addition of SDS sample buffer. Both cellular samples were pooled together in SDS sample buffer separated by 12% SDS/PAGE (1×10^4 cells/lane) and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween-20) for 1 h, and incubated overnight at 4 °C with a rabbit anti-human polyclonal antibody against Fas, Fas ligand and caspase-3 (which reacts with both the 32 kDa

proenzyme and the spliced 20 kDa active form of caspase-3), with a goat anti-human polyclonal antibody against procaspase-3, diluted 1:500 in blocking buffer, or with blocking buffer only (negative control). After washing three times with TTBS, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Fas, Fas ligand, caspase-3) diluted 1:10 000 or rabbit anti-goat (procaspase-3) diluted 1:5000 in blocking buffer. The presence of antibody-protein complexes was detected by enhanced chemiluminescence. A human fibroblast lysate in SDS/PAGE buffer (Becton Dickinson, Franklin Lakes, NJ, USA) was used as a positive control and bands were detected at the appropriate sizes. In the absence of the primary antibodies no specific bands were detected on the blot.

Cell death analysis

Apoptotic cells were identified by analysis of phosphatidylserine (PS) at the outer leaflet of apoptotic cell membranes using annexin-V and propidium iodide (Vermes *et al.* 1995). Cells were seeded at a density of 2.5×10^4 cells/well in 24-multiwell culture dishes and cultured overnight, followed by treatment in serum-free culture medium supplemented with or without an agonistic mouse anti-human Fas IgM (50 ng/ml clone CH11) at various times in the absence or presence of the following compounds: Z-VAD-FMK (10^{-5} M), LH (0.1, 1 or 10 ng/ml), 8-CPT-cAMP (10^{-6} M), H89 (10^{-6} M), PMA (10^{-6} M), inactive phorbol ester (10^{-6} M) or H7 (10^{-6} M). Detached cells were retained by centrifugation of the culture medium for 5 min at 6000 g and adhesive cells were harvested after 5 min of trypsinization. Adhesive and floating cells were pooled together and were incubated with annexin-V-fluorescein (dilution 1:200) containing propidium iodide (dilution 1:500) in Hepes buffer for 10 min in the dark. The percentage of annexin-V-labeled cells was measured per 7500 cells and quantified by flow cytometry, using a FACScan flow cytometer (Becton Dickinson).

Cell growth analysis

The dose-response effect of LH and 8-CPT-cAMP on the proliferative activity of the ovarian cancer cell lines was assessed by a BrdU incorporation assay using a 5-bromo-2'-deoxy-uridine labeling and detection kit. BrdU is an analog of thymidine that is incorporated into the DNA of cells in the S phase of the cell cycle. Cells, seeded at a density of 2.5×10^4 cells/well, were grown on glass coverslips placed in 24-multiwell culture dishes in culture medium. After culture overnight the medium was discarded and replaced by serum-free culture medium with or without supplementation of LH (0.1, 1 or 10 ng/ml) or 10^{-6} M 8-CPT-cAMP, followed by another culture period of 8 or 24 h. Three hours prior to the end of each

culture period, BrdU was added to the culture medium in a final concentration of 3 µg/ml. Cells were fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) for 10 min at room temperature. After fixation the cells were rinsed with 70% ethanol, PBS and finally distilled water. The cells were pretreated with 1% periodic acid at 55 °C for 20 min, followed by immersion in respectively tap water and PBS. Next, the cells were blocked for 10 min with 5% BSA diluted in PBS and then incubated for 1 h with a mouse anti-BrdU antibody diluted 1:100 in PBS containing 0.05% acetylated BSA. The coverslips were again washed in PBS and incubated for 1 h with a goat anti-mouse IgG horseradish peroxidase-labeled antibody diluted 1:100 in PBS containing 0.05% acetylated BSA. After this incubation the cells were washed in PBS and bound antibody was visualized after the addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride in PBS to which 1% nickel aminosulphate and 0.05% H₂O₂ were added. The cells were subsequently counterstained with Mayer's hematoxylin. Black staining identified BrdU incorporation into the nuclei.

To examine the specificity of the immunostaining, control coverslips were incubated with normal mouse serum instead of primary antibody and similarly processed. No staining was observed in these controls. HEY cells cultured in the presence of serum were used as a positive control and processed as described above. The percentage of BrdU-labeled cells was determined by counting 500 (labeled and unlabeled) cells per coverslip under a light microscope. Values are presented as means ± S.E.M. from one representative experiment using quadruplicates. Experiments were repeated three times with similar results.

cAMP enzyme-immunoassay

To measure total cAMP levels (intracellular and secreted in the medium), cells (2.5×10^4 cells/well) were plated in 24-multiwell dishes and grown overnight in culture medium. The medium was discarded and cells were pre-incubated in serum-free culture medium for 1 h and then treated with LH (0.1 and 10 ng/ml) for 60 min in the presence of 1 mM IBMX. Incubations without IBMX were below the detection level of the assay used. Therefore, all cAMP measurements were performed in the presence of IBMX, which resulted in a significant increase in basal cAMP levels, making a stimulus detectable. Treatment with IBMX (1 mM) alone was used as a control. Total cAMP levels (present in both the medium and within the cells) were measured using a cAMP Biotrak[®] competitive enzyme-immunoassay (EIA) system (GE Healthcare, Eindhoven, The Netherlands), according to the manufacturer's protocol.

Statistical analysis

Statistics was performed by a one-way analysis of variance (ANOVA), unless otherwise mentioned. Differences between group variances were determined with Tukey's multiple comparison test. Values were considered to be statistically significantly different when $P < 0.05$.

Results

Presence of Fas, Fas ligand and procaspase-3 in HEY cells

To investigate whether LH affects Fas-induced apoptosis in OSE cells, we first demonstrated the presence of the Fas pathway in HEY cells. Fas receptor and signaling molecules were detected by both Western blot analysis and immunohistochemistry. In HEY cells Fas receptor (45 kDa), Fas ligand (40 kDa) and procaspase-3 (32 kDa) proteins were observed to be present under basal conditions (Fig. 1). Fas immunostaining resulted in staining of an additional protein band of approximately 40 kDa, which has also been reported by Kamitani *et al.* (1997).

Activation of Fas induces apoptosis in HEY cells

To determine whether the presence of Fas, Fas ligand and procaspase-3 in HEY cells was accompanied by a functional Fas pathway, cells were treated with an agonistic anti-Fas receptor antibody (CH11). Significant apoptosis occurred in HEY cells upon CH11 treatment. Dramatic changes in cell morphology, characteristic for apoptosis, were observed in response to treatment with 50 ng/ml CH11 (Fig. 2A). Cells shrank, rounded up, showed blebbing in quick succession, and detached from the culture dish in a time- and concentration-dependent manner (data not shown). In addition, Fas receptor activation also resulted in splicing of procaspase-3 and the immunocytochemical detection of active caspase-3 and PARP cleavage fragments (Fig. 2A), indicating that CH11 induced apoptosis in these cells. Caspase-3 activation was further confirmed by Western blot analysis (Fig. 2B). This stimulatory effect on caspase-3 activity was completely reversed by co-treatment with Z-VAD-FMK, a well-known inhibitor of caspase-3 processing (Fig. 2B). Fas-induced apoptosis resulted in a significant translocation of PS to the outer leaflet of the plasma membrane as quantified by FACS analysis using annexin-V/propidium iodide double staining (Fig. 2C). The number of cells displaying PS at the outer leaflet of the plasma membranes increased over time with 12.1 ± 2.4 , 18.3 ± 2.7 and $44.1 \pm 4.2\%$ of HEY cells staining positive for annexin following 6, 12 and 24 h of CH11 treatment, respectively (Fig. 2D). Viable cells that lacked annexin staining remained in culture even after 72 h, indicating that not all HEY cells became apoptotic during CH11 treatment (data not shown). Z-VAD-FMK administration significantly

Figure 1 Detection of Fas, Fas ligand and procaspase-3 protein in HEY cells. HEY cells (5×10^4 cells/ml) were maintained overnight and subsequently serum and growth factor starved for 24 h. Fas, Fas ligand (FasL) and procaspase-3 protein levels were estimated by Western blotting (A) and immunohistochemistry (B) as described in the Materials and Methods section. Immunopositive cells showed brown-stained cytoplasm. The control represents a staining in which the polyclonal primary antibody was replaced by normal rabbit serum. A representative experiment is shown, which was repeated three times with similar results.

inhibited Fas-induced PS externalization within the time frame studied to similar levels ($P < 0.01$), as observed in controls (Fig. 2C–D).

Presence of LH-R and LH responsiveness in HEY cells

Under basal culture conditions LH-R protein could be detected in HEY cells. LH-R immunostaining was detected intracellularly and on the plasma membrane of HEY cells (Fig. 3A). To test whether the LH-R was responsive to LH in these cells, HEY cells were treated without or with LH (0.1 and 10 ng/ml) in the presence of IBMX (1 mM), a phosphodiesterase inhibitor, and total cAMP levels were measured. Treatment with 0.1 and 10 ng/ml LH resulted respectively in a 1.3- and 1.4-fold

increase in total cAMP levels compared with the IBMX control after 60 min of incubation (Fig. 3B).

LH promotes cell survival by suppressing Fas-induced apoptosis in HEY cells

Treatment of HEY cell cultures with LH resulted in a significant inhibition of CH11-induced apoptosis in a concentration-dependent manner as measured by FACS analysis, using annexin-V/propidium iodide double staining. A representative experiment is shown in Fig. 4A. When treated with low doses of LH (0.1 or 1 ng/ml) for 12 h, the percentage of apoptotic cells was significantly reduced by respectively 22.7 ± 5.3 ($n=7$) and $29.2 \pm 3.1\%$ ($n=11$) compared with cells treated with CH11 alone (Fig. 4B). Z-VAD-FMK inhibited Fas-induced apoptosis (for a representative experiment see Fig. 2D). Treatment with a higher dose (10 ng/ml) had no modulating effect on apoptosis in HEY cells. The anti-apoptotic effect of LH observed at 12 h of culture was transient, as after 24 h of incubation the amount of Fas-induced apoptosis was similar to cells cultured in the absence of LH (Fig. 4A). A similar trend was observed when apoptosis was determined by flow cytometry by measuring DNA content in permeabilized cells (data not shown) as described by Guthrie *et al.* (1998). The level of apoptosis was somewhat lower with this assay compared with the annexin-V assay. This was not unexpected as DNA fragmentation is a late apoptotic event and the detection of PS at the outer plasma membrane leaflet by annexin-V is a relatively early apoptotic marker.

LH does not promote OSE cell survival by cell proliferation in HEY cells

To exclude the possibility that the anti-apoptotic effect of LH was actually caused by increased cell growth, we measured incorporation of BrdU by HEY cells in the absence of serum. HEY cells proliferated under these serum-free culture conditions. The number of cells that incorporated BrdU decreased over time with 28.7 ± 0.8 and $7.8 \pm 1.2\%$ of HEY cells staining positive for BrdU following 8 and 24 h of culture, respectively ($n=3$). LH did not change the levels of BrdU incorporation in HEY cells. After 8 h of incubation, the percentage of BrdU-labeled cells in 0.1, 1 and 10 ng/ml LH-treated HEY cells was 29.2 ± 0.9 , 29.3 ± 1.3 and $29.3 \pm 1.3\%$, respectively, versus 8.1 ± 0.8 , 8.2 ± 1.1 and $8.3 \pm 1.1\%$ following 24 h of culture.

LH also suppresses Fas-induced apoptosis in Caov-3 cells

To test whether the suppressive effect of LH on Fas-induced apoptosis in HEY cells was not a cell-line-specific effect; we also assessed the presence, activation and modulation of the Fas pathway in Caov-3 cells. Fas receptor,

Figure 3 Presence of functional LH-Rs in HEY cells. (A) HEY cells (5×10^4 cells/ml) were maintained overnight, then serum-starved for 24 h and LH-R protein expression was determined by immunohistochemistry as described in the Materials and Methods section. The cytoplasm of cells stained positively with the LH-R antibody was brown. (B) HEY cells were incubated in serum-free medium for 1 h and then further cultured in the absence (0 ng/ml LH) or presence (0.1 or 10 ng/ml) of LH and 1 mM IBMX for 60 min. Total cAMP levels (intracellular and secreted in the medium) were measured as described in the Materials and Methods section. Values are expressed as means $\pm$ S.E.M. of quadruplicate incubations from one representative experiment. The experiment was repeated twice. A Student's *t*-test was used to compare the mean of cAMP levels between the control group and the groups treated with LH. Significantly different from control (IBMX+0 ng/ml LH), * $P < 0.05$.

Fas ligand and procaspase-3 were present in Caov-3 cells under basal conditions as detected by both Western blot analysis and immunocytochemistry (Fig. 5A and B). The Caov-3 cell line was also responsive to Fas activation although the degree of Fas-induced cell death varied. The level of Caov-3 cells that stained positive for annexin-V following 12 h of CH11 administration was 2-fold lower compared with HEY cells (compare Fig. 5C with Fig. 4B). Despite differences in the number of apoptotic cells between the two ovarian cancer cell lines, the transient anti-apoptotic effect of LH was very similar in both cell lines. However, due to the relative resistance of Caov-3 cells to CH11 within the studied time frame, we decided to carry out the remainder of the experiments in HEY cells only.

The anti-apoptotic effect of LH on Fas-induced apoptosis was mimicked by PKA activation and reversed by blocking of PKA activity in HEY cells

In steroid-producing ovarian follicular cells, the binding of LH to its cognate receptor stimulates adenylate cyclase, resulting in subsequent production of cAMP and activation of PKA (Hunzicker-Dunn & Birnbaumer 1985, Cooke 1999). To investigate whether the LH anti-apoptotic pathway in OSE cancer cells includes cAMP/PKA, we tested whether addition of a cell-membrane-permeable cAMP analog (8-CPT-cAMP) mimicked the anti-apoptotic effect of LH in HEY cells. Addition of 10^{-6} M 8-CPT-cAMP has been demonstrated in other cell-culture systems to mimic the effects of LH on steroidogenesis (Cooke 1999).

Figure 6 shows the effects of an activator and inhibitor of PKA activity on Fas-induced apoptosis in HEY cells. The percentage of Fas-induced apoptotic cells was reduced significantly by $37.0 \pm 2.0\%$ ($P < 0.01$) following 8 h treatment with 8-CPT-cAMP (10^{-6} M). This reduction was similar to what was observed after treatment with 1 ng/ml LH ($42.1 \pm 3.7\%$; Fig. 6). No significant additive effects on the inhibition of Fas-induced apoptosis were observed after combined treatment of 10^{-6} M 8-CPT-cAMP and 1 ng/ml LH, compared with single LH or 8-CPT-cAMP treatment. The anti-apoptotic effect of 8-CPT-cAMP was not due to increased cell growth,

Figure 2 Effect of Fas stimulation on various parameters indicative for apoptosis in HEY cells. (A) HEY cells (5×10^4 cells/ml) were maintained overnight and then treated for 8 h without or with an agonistic anti-Fas antibody (50 ng/ml CH11) in serum-free medium. The left of panel A shows phase-contrast images of non-stimulated (control) and CH11-stimulated HEY cells. The right panel shows immunohistochemical staining for active caspase-3 and PARP p85 in CH11-treated HEY cells. Arrows indicate apoptotic cells. (B) HEY cells were treated without or with 50 ng/ml CH11 in the presence or absence of 10^{-5} M Z-VAD-FMK for 4, 8 and 24 h. The presence of procaspase-3 (32 kDa) and active caspase-3 (20 kDa) was determined in whole cell lysates as described in the Materials and Methods section. (C) PS exposure was determined by FACS analysis at various times after treating cells without additions (control), 50 ng/ml CH11 or 50 ng/ml CH11 plus 10^{-5} M Z-VAD-FMK as described in the Materials and Methods section. Values on top of the right quadrant are the sum of the relative number of cells positive for annexin-V. Data shown are from one representative experiment, which was repeated three times with similar results. (D) Quantification of Fas-induced apoptosis in HEY cells following treatment with 50 ng/ml CH11 in the absence (open bars) or presence of 10^{-5} M Z-VAD-FMK (closed bars) as described for panel C. Values shown are means $\pm$ S.E.M. from four independent experiments. Significantly different from CH11-treated cells, ** $P < 0.01$.

Figure 4 Effect of LH on Fas-induced apoptosis in HEY cells. (A) FACS analysis of apoptosis in HEY cells after incubation in serum-free medium in the absence (control) or presence of 1 ng/ml LH and/or 50 ng/ml CH11 for 12 and 24 h. Values on top of the right quadrant are the sum of the relative number of cells positive for annexin-V. Data shown are from a representative experiment. (B) Quantification of Fas-induced apoptosis in HEY cells 12 h after treatment with various concentrations of LH as shown. Apoptotic indices represent the percentage of positively labeled annexin-V cells minus its respective non-treated control, which were determined as described for panel A. Values shown are means $\pm$ S.E.M. from eight independent experiments. Significantly different from CH11-treated cells, ** $P < 0.01$.

as no significant differences were observed in the percentage of BrdU-labeled HEY cells in the absence or presence of 10^{-6} M 8-CPT-cAMP (respectively 28.7 ± 0.8 and $30.7 \pm 0.9\%$). The effect of the cAMP analog was, however, biphasic. In contrast to the inhibitory dose of 10^{-6} M 8-CPT-cAMP, no effect was observed in HEY cells treated with 10^{-5} M 8-CPT-cAMP while a high dose of 8-CPT-cAMP (10^{-4} M) significantly enhanced Fas-induced apoptosis by approximately 40%. High doses of cAMP analogs have been shown before to induce or accelerate apoptosis in various human cancer cell lines (Kim *et al.* 2001). Moreover, BrdU incorporation was also significantly increased in HEY cells treated with 10^{-4} M 8-CPT-cAMP (34.7 ± 1.2 versus $28.7 \pm 0.8\%$ in non-treated cells ($P < 0.05$)), suggesting an enhanced cell turnover in the presence of high concentrations of cAMP analogs.

Furthermore, we have investigated whether an inhibitor of PKA activity (H89) could antagonize the anti-apoptotic effect of LH in HEY cells (Fig. 6). H89 by itself did not affect the level of apoptosis (data not shown), but the anti-apoptotic effect of LH (Fig. 6) and of 10^{-6} M 8-CPT-cAMP (data not shown) on Fas-induced apoptosis was almost completely reversed when 10^{-6} M H89 was added to the culture medium.

To investigate the possibility whether LH also signals via PKC, HEY cells were treated with an activator and inhibitor of PKC activity (Fig. 6). In the presence of the phorbol ester PMA (10^{-6} M), a stimulator of PKC activity, the number of Fas-induced apoptotic cells was significantly reduced compared with untreated Fas-stimulated cells, while no significant additive effect on the inhibition of Fas-induced apoptosis was observed after combined treatment with LH and PMA. Treatment with

Figure 5 Presence, activation and modulation of the Fas pathway in Caov-3 cells. Caov-3 cells (5×10^4 cells/ml) were maintained overnight and subsequently serum and growth factor starved for 24 h. Fas, Fas ligand (FasL) and procaspase-3 protein levels were estimated by Western blotting (A) and immunohistochemistry (B) as described in the Materials and Methods section. Immunopositive cells showed a brown cytoplasm. The control represents a staining in which the primary polyclonal antibody was replaced by normal rabbit serum. A representative experiment is shown, which was repeated three times with similar results. (C) PS exposure was determined by FACS analysis after treating cells with 50 ng/ml CH11 in the absence or presence of various concentrations of LH as shown for 12 h as described in the legend of Fig. 4. Significantly different from CH11-treated cells, ** $P < 0.01$ or * $P < 0.05$.

an inactive phorbol ester (10^{-6} M) in either the absence or presence of LH did not affect the number of Fas-induced apoptotic cells. Additionally, treatment with H7 (10^{-6} M), an inhibitor of PKC activity, by itself did not affect the level of apoptosis (data not shown). H7 only partially prevented the anti-apoptotic effect of LH in HEY cells (Fig. 6). Moreover, no significant differences were observed between CH11-stimulated HEY cells treated with LH in the absence or presence of H7. Thus, the anti-apoptotic effect of LH on CH11-induced apoptosis was mimicked by PKC activation, but not completely reversed by blockade of PKC activity. Although the involvement of PKC cannot be completely excluded, our observations suggest that LH exerts its anti-apoptotic effect predominantly through activation of the cAMP/PKA system.

Discussion

The results of the present study suggest a direct effect of LH on the survival of the OSE cancer cell lines HEY

and Caov-3 following Fas-receptor activation. OSE cells expressed both functional LH and Fas receptors; addition of LH resulted in cAMP production, suggesting that the LH-R is functional, while stimulation of the Fas receptor induced apoptosis in these cells. LH did not affect cell proliferation in the absence of serum and growth factors, but affected OSE cell survival through the inhibition of Fas-induced apoptosis. This anti-apoptotic effect of LH could be mimicked by a membrane-permeable cAMP analog (8-CPT-cAMP), while treatment with a PKA inhibitor (H89) reversed the anti-apoptotic effect of LH. These observations suggest that LH exerts its anti-apoptotic effect predominantly through activation of PKA.

The Fas system has been shown to play a major role in regulating ovarian homeostasis by triggering apoptosis in various ovarian cell types, such as granulosa and theca cells (Tilly 1996, Ghahremani *et al.* 1998, Foghi *et al.* 1998). A role for Fas-induced apoptosis has also been implicated in the normal OSE. OSE cells undergo apoptosis to facilitate ovulation, as demonstrated in the goat (Murdoch & McDonnell 2002). This apoptotic process presumably

Figure 6 Effect of PKA/PKC signaling on Fas-induced apoptosis. HEY cells were treated for 8 h with 50 ng/ml CH11 (open bars) or 50 ng/ml CH11 plus 1 ng/ml LH (closed bars) in the absence or presence of various compounds: 10^{-6} M 8-CPT-cAMP ($n=8$), 10^{-6} M H89 ($n=8$), 10^{-6} M PMA ($n=8$), 10^{-6} M inactive phorbol ester ($n=6$) or 10^{-6} M H7 ($n=4$). Apoptotic indices were determined by FACS analysis and quantified as described in the legend of Fig. 4. Values represent the means $\pm$ S.E.M. from four to eight independent experiments. Significantly different from cells treated with CH11 only, ** $P<0.01$ or * $P<0.05$.

takes place via activation of the Fas pathway, as OSE cells at the ovulatory site expressed Fas, Fas ligand and active caspase-3 (Slot *et al.* 2006a). The Fas apoptotic machinery is also present in OSE cells at the tumor site and in OSE cells lining irregular inclusions cysts which are thought to have, under certain conditions, the capacity to develop into epithelial cancers (Scully 1995). Despite the presence of the Fas pathway, apoptotic cells were relatively scarce in OSE tumors (Slot *et al.* 2006b).

Fas and Fas ligand expression in HEY and Caov-3 cells are in accordance with previously published data in OSE tumor cells (Ghahremani *et al.* 1998, Ben Hur *et al.* 1999, Baldwin *et al.* 1999, Das *et al.* 2000, Munakata *et al.* 2000). The presence of the Fas pathway seems to reflect the ability of OSE cancer cell lines to undergo Fas-induced apoptosis. The degree of Fas-induced cell death may vary, as we found low numbers of apoptosis and immunostaining for Fas, Fas ligand and procaspase-3 in another OSE cell line, Caov-3 cells, while abundant expression of these antigens and much higher sensitivity to Fas activation were found in the HEY cell line. Dysregulation of the Fas system may contribute to ovarian tumor development and progression as has been suggested by Ghahremari and colleagues (1999). Nevertheless, the mechanism by which apoptosis is inhibited and the survival factors involved remain to be determined.

Gonadotrophins have been reported to play a major role in the regulation of ovarian apoptosis. It has been shown that follicle-stimulating hormone or the LH analog hCG suppressed the spontaneous onset of follicular DNA fragmentation in serum-free cultures of rat

preovulatory follicles (Chun *et al.* 1994). Furthermore, treatment of hypophysectomized immature rats with follicle-stimulating hormone decreased granulosa cell apoptosis *in vivo* (Chun & Hsueh 1998). Despite these observations, not much is known about the effects of gonadotrophins on OSE cell growth and/or survival. In the present study, we demonstrated that activation of Fas-induced apoptosis in the OSE cancer cell lines HEY and Caov-3 could be blocked, in part, by treatment for 12 h with the gonadotrophin LH, in a relatively low concentration of 0.1–1.0 ng/ml. In granulosa cells it has also been observed that high doses of gonadotrophic hormones, though inducing a short-lasting cAMP increase, had no inhibitory effect on apoptosis, as was found when lower doses were used (Aharoni *et al.* 1995). The observation that 10 ng/ml LH did not inhibit Fas-induced apoptosis OSE cells may be caused by desensitization of the LH-R (Hunzicker-Dunn & Birnbaumer 1985). Doses of LH, in the range of 10–100 ng/ml, initially causing an increase in cAMP, have been described to induce a rapid desensitization of the LH-R in different cell systems (Wang *et al.* 1991, Ulaner *et al.* 1999). A similar transient inhibitory effect on Fas-induced apoptosis was observed in response to a membrane-permeable cAMP analog, which is in line with some previous studies (Parvathenani *et al.* 1998, Niwa *et al.* 1999). cAMP might act upstream of caspase-3 activation to delay the activation of the apoptotic pathway as has been postulated in human neutrophils (Parvathenani *et al.* 1998, Niwa *et al.* 1999). Eventually, such an anti-apoptotic block may be overruled by other death signaling pathways.

A delay in cell death as induced by LH may under certain circumstances increase the chance of survival of mutated OSE cancer cells *in vivo*. Moreover, it might change the ability of cancer cells to respond to chemotherapeutic agents. For example, cisplatin, which is the preferred chemotherapeutic agent for treatment of OSE cancer, has been shown to sensitize cancer cells to Fas-mediated apoptosis, by modulating several components of this apoptotic pathway. In ovarian cancer cell lines, cisplatin can upregulate Fas and Fas ligand (Uslu *et al.* 1996, Wakahara *et al.* 1997), resulting in caspase-3 activation and eventually apoptosis. Moreover, it was demonstrated that the LH homolog hCG lowered the chemosensitivity to the anticancer drug cisplatin in the OSE cancer cell line OVCAR-3 (Kuroda *et al.* 1998). These authors hypothesized that the hCG-induced inhibition of cisplatin-induced apoptosis was dependent on the presence of LH-Rs, as in cells lacking functional LH-Rs, i.e. the SKOV-3 cell line, hCG failed to block cisplatin-induced cell death (Kuroda *et al.* 1998).

To obtain more insight in the role of LH in the inhibition of Fas-induced apoptosis in OSE cells, we have investigated whether the survival effect was indeed the result of inhibition of apoptosis or was caused by stimulation of HEY cell growth. Several studies have demonstrated that certain OSE cancer cell lines undergo enhanced cellular proliferation following LH addition (Casagrande *et al.* 1979, Simon *et al.* 1983, Osterholzer *et al.* 1985, Wimalasena *et al.* 1992, Syed *et al.* 2001, Parrott *et al.* 2001b). Controversy, however, exists whether LH has an influence on the proliferative activity of all OSE cells, as also decreased (Zheng *et al.* 2000, Tourgeman *et al.* 2002) or unchanged (Ala-Fossi *et al.* 1999, Ivarsson *et al.* 2001, Wright *et al.* 2002) OSE cell proliferation in response to LH have been reported. In the HEY cell line, proliferation rates as measured by the use of a BrdU-incorporation assay, were not affected following supplementation with LH under serum- and growth factor-free conditions. This suggests that the observed increased OSE survival was due to inhibition of apoptosis and not to a modulation of cell proliferation.

We have also analyzed the signal transduction pathway of LH in these cells. In ovarian follicular cells, it is well established that LH interacts with its cognate receptor and stimulates a G_s -protein that leads to the production of cAMP, followed by activation of PKA and subsequently induces steroid production. Interestingly, the second-messenger molecule cAMP also modulates the apoptotic program in these cells; when follicles were treated *in vitro* with low doses of cAMP analogs, apoptosis was prevented (Johnson *et al.* 1996, Chun & Hsueh 1998). In non-follicular cells, cAMP and cAMP-dependent PKA have been shown to modulate apoptosis in a wide variety of ways. In human neutrophils, cAMP analogs activate PKA resulting in a delay or suppression of apoptosis induced by either tumor necrosis factor- α or Fas. Inhibitors of

PKA activity could antagonize the suppressive effect of cAMP on tumor necrosis factor- α -induced apoptosis (Parvathenani *et al.* 1998, Niwa *et al.* 1999, Martin *et al.* 2001). In contrast, in various human cancer cells, cAMP analogs have been demonstrated to induce or accelerate apoptosis (Kim *et al.* 2001).

Up to now, there was no evidence for activation of PKA in OSE cancer cells, nor for regulation of PKA activity by LH in these cells. In the present study, we have demonstrated that HEY cells respond to LH with enhanced cAMP production. Moreover, we showed that a cAMP analog could mimic the anti-apoptotic effect of LH, and that an inhibitor of PKA could reverse this effect. Previously, it has been demonstrated that the extent and the duration of the intracellular rise in cAMP might play an important role in controlling the rate and extent of apoptosis in cultured granulosa cells (Aharoni *et al.* 1995). In addition, very low doses of LH have been shown to elicit physiological actions in a variety of cells concomitant with barely detectable cAMP responses (Browne & Bhalla 1991, Yong *et al.* 1994). A small but significant 15% increase in cAMP levels in a lung cancer cell line was able to inhibit UV-induced apoptosis (Hastings *et al.* 2004). Possibly, the anti-apoptotic effect of LH is exerted by small increases of cAMP concentrations acting at discrete cellular locations and by activation of multiple signaling pathways in addition to PKA, such as the PKC pathway (Johnson 2003). Activation of PKC by treatment with the phorbol ester PMA has been demonstrated to block Fas-induced apoptosis in human T-cells and Jurkat cells (Rudert *et al.* 1997). The present study demonstrates that Fas-induced apoptosis is also blocked by PKC activation in the OSE cell line HEY. However, the PKC inhibitor H7 could not completely antagonize the effect of LH, implying that the anti-apoptotic effect of LH most likely does not signal predominantly through PKC.

Gonadotrophins have been suggested to play a role in the etiology of OSE cancer, as several cases have been reported of OSE cancers arising in infertile women during or after prolonged treatment with gonadotrophins. Moreover, the incidence of OSE cancer is increased in women during the years after menopause when serum gonadotrophin levels are high, i.e. within the range of 7–47 IU/l (Miller 2004), which corresponds to approximately 1–7 ng/ml LH. Hence, the transient anti-apoptotic effect of LH in OSE cancer cells as demonstrated in the present study may be relevant for future treatment of OSE. However, in order to be able to extrapolate our *in vitro* results to the *in vivo* situation further research is required.

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