Abstract. The elevation of Luteinizing hormone (LH) is commonly observed in epithelial ovarian cancer. This correlation suggests a causal relationship between LH and ovarian cancer. LH has been reported to inhibit apoptosis in ovarian cancer cells. Programmed cell death gene 6 (PDCD6), also known as apoptosis-linked gene-2, is an apoptotic mediator that is required for apoptosis to numerous death stimuli. Therefore, the aim of the present study was to determine whether PDCD6 may be induced by LH in ovarian cancer, and whether LH may affect the apoptosis through PDCD6. Flow cytometry was used to detect the effects of cisplatin on the induction of apoptosis by LH. PDCD6 expression was monitored by quantitative polymerase chain reaction and western blotting. The signaling transduction pathways were also investigated by western blotting. The present study demonstrated that LH reduced cisplatin-induced apoptosis in ovarian OVCAR-3 and SKOV-3 cancer cells. The results indicated that PDCD6 expression was inhibited by LH. In addition, the inhibition of PDCD6, induced by LH, was mediated through the activation of the phosphatidylinositol 3-kinase/protein kinase B and p44/42 mitogen-activated protein kinase transduction signaling pathways. The present results suggest that LH affects the sensitivity of ovarian cancer cells to chemotherapy, primarily by signaling to inhibit apoptosis and to additionally suppress PDCD6.

Introduction

Ovarian cancer ranks fifth overall for cancer-associated mortalities in women, with lung, breast, colorectal and pancreatic cancer ranking 1-4, respectively, and accounts for 5% of all cancer-associated mortalities in women (1). The overall five-year survival rate is ~44%, and only 27% for patients that are at the distant stage of the disease at diagnosis (1). The exact cause of epithelial ovarian cancer has not yet been determined, although follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are understood to be associated with ovarian malignancy (2). With apoptosis and apoptosis-linked genes demonstrating key roles in ovarian cancer tumorigenesis (3,4), it is important to note that FSH has been previously reported to inhibit apoptosis in ovarian cancer cells (5). However, little is currently understood regarding the association between LH and apoptosis in ovarian cancer, with further investigation required.

Programmed cell death gene 6 (PDCD6), also known as apoptosis-linked gene-2, is an apoptotic mediator that is required for apoptosis to occur in response to numerous death stimuli (6). It has recently been suggested that PDCD6 is an independent predictor of progression-free survival in patients with epithelial ovarian cancer (7). However, its effect on ovarian cancer apoptosis is currently unknown.

To understand the function and regulation of LH on ovarian cancer apoptosis, the present study investigated the effect of LH on apoptosis in vitro using flow cytometry. The protein and mRNA expression levels of PDCD6 were analyzed using western blotting and quantitative polymerase chain reaction (qPCR). The signal transduction pathways were also examined using western blotting.

Materials and methods

Reagents and antibodies. LH, SP600125 [a stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK) inhibitor], SB203580 [a p38 mitogen-activated protein kinase (MAPK) inhibitor], LY294002 [a phosphatidylinositol 3-kinase (PI3K) inhibitor] and U0126 [a p44/p42 MAPK (extracellular signal-regulated kinase 1/2; Erk1/2) inhibitor] were acquired from Sigma-Aldrich (St. Louis, MO, USA). The PDCD6 rabbit anti-human polyclonal antibody (cat. no. 12303-1-AP; dilution, 1:1,000) was purchased from Proteintech Group, Inc. (Chicago, IL, USA) for western blotting. The antibodies against phospho-protein kinase B (pAKT; rabbit anti-human monoclonal antibody; cat. no. 4060; dilution, 1:1,000), protein kinase B (AKT; rabbit anti-human monoclonal antibody; cat. no. 9272; dilution, 1:1,000), phospho-c-Jun (pJUN; rabbit
INHIBITS APOPTOSIS IN OVARIAN CANCER CELLS were as follows: 42 (µM), incubations were performed Takara Bio, Inc. (Otsu, Japan), /polyacrylamide Danvers, MA, USA). B2˚ and immunoblotted with /: PDCD6, 5'-GGA TGA TCG ATA AGA ACG AGC -polyacrylamide. The apoptotic cells were determined using Annexin V-FITC Apoptosis detection kit. *Pc<0.05 vs. cisplatin-treated SKOV-3 cells and **P<0.05 vs. cisplatin-treated OVCAR-3 cells. LH, luteinizing hormone; PDCD6, programmed cell death gene 6; CON, control.

Culture. The ovarian cancer cell lines, OVCAR-3 and SKOV-3, were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the company’s protocols. SKOV-3 cells were cultured at 37°C with 5% CO2 in McCoy’s 5a medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 0.1% gentamicin sulfate (Gemini Bio Products, West Sacramento, CA, USA), while OVCAR-3 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 0.1% gentamicin sulfate. All experiments were performed with cell lines at 60% to 80% confluence. To investigate the expression of PDCD6, the cells were treated for up to 24 h with LH (40 U/l) in the absence of FBS. To investigate signal transduction, the cells were treated for up to 120 min with LH (40 U/l) in the absence of FBS. To evaluate the effects of the specific inhibitors, the cells were pre-treated with each inhibitor for 30 min, and LH was then added for an additional 30 min or 24 h without FBS.

Apoptosis analysis. Following a 48-h incubation with LH, with or without cisplatin (10 µM), the number of apoptotic cells was determined using the Annexin V-FITC Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA) followed by flow cytometry. The apoptotic cells were determined using a FACScan cytofluorometer from BD Biosciences (Franklin Lakes, NJ, USA) with Cell Quest software version 5.1, also from BD Biosciences. The early apoptotic [Annexin-V positive, propidium iodide (PI) negative] and late apoptotic (Annexin-V positive, PI positive) cells were included in cell death determinations.

qPCR. Total RNA was prepared using the RNAprep Pure Cell kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer’s protocols. The primers utilized for the SYBR Green (Tiangen Biotech Co., Ltd.) qPCR were as follows: PDCD6, 5'-GGATGATCGATAAGACGGACG TGAA-3' (forward) and 5'-ATGAGGATGTCGGGAAC TGGGTC-3' (reverse); and GAPDH, 5'-ATGAAATCTCCCA TCACCATCTT-3' (forward) and 5'-CGCCCCACTTGATT TGGAG-3' (reverse). The reaction mixture was composed of 12.5 µl One Step SYBR RT-PCR Buffer III, 0.5 µl Takara Ex Taq HS DNA Polymerase (5 U/µl), 0.5 µl PrimeScript RT Enzyme Mix II, 0.5 µl Forward PCR Primer (10 µM), 0.5 µl Reverse PCR Primer (10 µM), 2 µl total RNA (100 ng) and 8.5 µl RNase-Free dH2O (Thermo Fisher Scientific, Inc.), all obtained from Takara Bio, Inc. (Otsu, Japan). The qPCR conditions in an Applied Biosystems 7500 series qPCR system (Thermo Fisher Scientific, Inc.) were as follows: 42°C for 5 min, followed by 95°C for 10 sec, then 40 cycles of 95°C for 5 sec and 60°C for 30 sec. RNase-Free dH2O without RNA was set as a negative control. The relative mRNA expression levels were calculated and normalized using the qPCR and the 2-ΔΔcq method.

Western blotting. Following cell lysis, 30 µg total protein was separated on 8-12% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Uppsala, Sweden) and immunoblotted with specific primary antibodies (PDCD6, GAPDH, pAKT, AKT, pJUN,c-Jun,phospho-p44/42MAPK,p38MAPK,phospho-p38 MAPK, pErk1/2, and Erk1/2) at 4°C overnight. Subsequently, secondary antibody (sheep anti-rabbit IgG secondary antibody; cat. no. KC-RB-035; dilution, 1:5,000; or sheep anti-mouse IgG secondary antibody; cat no. KC-MM-1302; dilution, 1:1,000; Kangchen Bioengineering Co.) incubations were performed.
at room temperature for 60 min. The signals were detected using the Amersham ECL Western Blotting Detection kit (GE Healthcare Life Sciences, Uppsala, Sweden). GAPDH was used for the loading control. Densitometry (Image J; National Institutes of Health, Bethesda, MD, USA) was used to assess the differences in the results.
**Results**

**Effect of LH on ovarian cancer cell apoptosis.** Due to the prevalence of ovarian cancer in post-menopausal women with ~40 U/l LH, this concentration was chosen for the present study (8). As presented in Fig. 1, LH treatment resulted in a slight decrease in the rate of apoptosis when compared with controls in the SKOV-3 and OVCAR-3 cells, however, the difference was not significant (P>0.05). To determine whether LH could block apoptosis induced by cisplatin, the SKOV-3 and OVCAR-3 cells were treated with 10 µM cisplatin, or a combination of 40 U/l LH and 10 µM cisplatin. As presented in Fig. 1, the rate of apoptosis induced by cisplatin was significantly suppressed by LH (P<0.05).

**PDCD6 expression is inhibited by LH.** The SKOV-3 and OVCAR-3 cells were treated with 40 U/l LH for up to 24 h. qPCR indicated that PDCD6 mRNA was significantly downregulated when treated with LH for 16 h (P<0.05; Fig. 2A). Additionally, western blotting analysis demonstrated that the protein expression of PDCD6 decreased when cells were treated with LH for 24 h (P<0.05) (Fig. 2B).

**Signaling transduction pathways induced by LH.** The present study investigated whether the SAPK/JNK, p38 MAPK, PI3K/AKT and Erk1/2 signaling pathways are involved in LH-mediated apoptosis. Due to LH exerting similar effects on each of the cell lines, signaling was only analyzed in the SKOV-3 cells. It was observed that LH induced the phosphorylation of AKT and Erk1/2 (P<0.05; Fig. 3), however, it did not affect the phosphorylation of p38 MAPK and c-Jun (P>0.05).

**LH-induced phosphorylation is blocked by inhibitors.** The SKOV-3 and OVCAR-3 cells were pre-treated with the inhibitors for 30 min, and LH was then added for an additional 30 min. A pre-treatment with LY294002 or U0126 blocked LH-induced phosphorylation (Fig. 4).

**The LH-induced PDCD6 protein inhibition is neutralized by the inhibitors.** The SKOV-3 and OVCAR-3 cells were pre-treated with the inhibitors for 30 min, and LH was then added for an additional 24 h. LY294002 and U0126 significantly neutralized LH-induced PDCD6 protein inhibition (P<0.05; Fig. 5), however, SP600125 and SB203580 did not have the same effect (P>0.05).

**Discussion**

The majority of ovarian tumors develop in post-menopausal women presenting with high gonadotropin levels. Thus, gonadotropins are regarded as possible risk factors for the formation of ovarian tumors (2). In 1992, Ohtani et al. (9) observed that FSH stimulated proliferation in ovarian cancer cells; subsequent to this, it was reported by Zheng et al. (10) that ovarian epithelial tumor growth was promoted by FSH and inhibited by LH. Choi et al. (11) demonstrated that gonadotropins upregulated epidermal growth factor receptors (EGFRs) through the activation of MAPK and PI3K in human ovarian surface epithelial cells. In a further study by Choi et al. (12), it was observed that gonadotropins activated proteolysis and increased invasion through the protein kinase A (PKA) and PI3K pathways in human epithelial ovarian cancer cells. Apoptosis serves an important role in the progression of ovarian cancer (13), and certain papers have suggested that human chorionic gonadotropin or LH may inhibit cisplatin-induced apoptosis in ovarian cancer cells (14,15).

PDCD6 is an important apoptotic mediator and a prognostic marker for gastric cancer (16); it is also an independent predictor of progression-free survival in patients with epithelial ovarian cancer (7). Park et al. (17) demonstrated that PDCD6 additively cooperated with anticancer drugs through the activation of nuclear factor-κB pathways. It was previously reported that FSH decreased PDCD6 levels and inhibited the rate of apoptosis in ovarian cancer cells (5). In the present study, it was observed that LH significantly blocked...
cisplatin-induced apoptosis in vitro. Furthermore, LH significantly reduced PDCD6 expression. Such results suggest that the downregulation of PDCD6, induced by LH, appears to be involved in the chemoresistance of ovarian cancer due to the induction of apoptosis inhibition.

Numerous studies have focused on the signaling pathways induced by gonadotropins in ovarian cancer cells and in human ovarian surface epithelium (OSE). Pon et al (18) reported that gonadotropins reduced N-cadherin expression in OSE cells through the PKA pathway. It has also been demonstrated that EGFR expression is regulated by gonadotropins via cyclic adenosine monophosphate, and this does not signal through PKA in immortalized OSE cells (19). FSH-induced DNA synthesis and proliferation may be neutralized by a number of protein kinase C (PKC) inhibitors, implying that the PKC pathway is also associated with FSH-induced cell growth (20).

It was previously reported that FSH induced vascular endothelial growth factor through the PI3K/AKT pathway (21). Slot et al (22) first demonstrated that LH protected HEY cells from Fas-induced apoptosis, occurring via a signaling cascade involving PKA. The Erk1/2 pathway has been identified to be involved in LH-induced survivin expression and apoptosis (15). A previous study indicated that FSH induced PDCD6 expression through activation of the PI3K/AKT and SAPK/JNK signaling pathways, and that treatment with LY294002, the specific PI3K inhibitor, antagonized the effects of FSH on pAKT and PDCD6 expression; furthermore, the study reported that treatment with SP600125, the specific SAPK/JNK inhibitor, antagonized the effects of FSH on pJUN and PDCD6 expression (5). It remains unknown whether FSH and LH regulate PDCD6 expression in ovarian cancer through different or identical signaling pathways. The results of the present study indicate that LH inhibits PDCD6 expression in ovarian cancer cells by activating the PI3K/AKT and Erk1/2 signaling pathways.

In conclusion, the current study demonstrated that LH inhibited cisplatin-induced apoptosis and suppressed PDCD6 expression in ovarian cancer cells. LH inhibited PDCD6 through the activation of the PI3K/AKT and Erk1/2 signaling pathways. The results suggest that LH serves a critical role in the chemoresistance of ovarian cancer based on the anti-apoptotic effect it induces.

References

1. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.
2. Mertens-Walker I, Baxter RC and Marsh DJ: Gonadotropin signalling in epithelial ovarian cancer. Cancer Lett 324: 152-159, 2012.
3. Dong Z, Yang L and Lai D: KLF5 strengthens drug resistance of ovarian cancer stem-like cells by regulating survivin expression. Cell Prolif 46: 425-435, 2013.
4. Duiker EW, Meijer A, van der Bilt AR, Meersma GJ, Kozi N, van der Zee AGJ, de Vries EG and de Jong S: Drug-induced caspase 8 upregulation sensitises cisplatin-resistant ovarian carcinoma cells to TRAIL-induced apoptosis. Br J Cancer 104: 1278-1287, 2011.
5. Huang Y, Jin H, Liu Y, Zhou J, Ding J, Cheng KW, Yu Y and Feng Y: FSH inhibits ovarian cancer cell apoptosis by up-regulating survivin and down-regulating PDCD6 and DR5. Endocrin Relat Cancer 18: 13-26, 2011.
6. Vito P, Lacanè E and D’Adamo L: Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer’s disease gene ALG-3. Science 271: 521-525, 1996.
7. So D, Xu H, Feng J, Jin Y, Gu X, Jing L, Katsaros D, Yu H, Xu S and Qi M: PDCD6 is an independent predictor of progression free survival in epithelial ovarian cancer. J Transl Med 10: 31, 2012.
8. Brodowska A, Laszczyńska M, Brodowski J, Masiuk M and Starczewska A: Analysis of pituitary gonadotropin concentration in blood serum and immunohistochemical localization of follicle stimulating hormone and luteinizing hormone receptors in ovaries of postmenopausal women. Histol Histopathol 27: 241-248, 2012.
9. Ohtani K, Sakamoto H and Satoh K: Stimulatory effects of follicular stimulating hormone on the proliferation of ovarian cancer cell line in vitro and in vivo. Nihon Sanka Fujinka Gakkai Zasshi 44: 717-724, 1992 (In Japanese).
10. Zheng W, Lu J, Luo F, Zheng Y, Feng Y, Felix JC, Lauchlan SC and Pike MC: Ovarian epithelial tumor growth promotion by follicle-stimulating hormone and inhibition of the effect by luteinizing hormone. Gynecol Oncol 76: 80-88, 2000.
11. Choi JH, Choi KC, Auersperg N and Leung PC: Gonadotropins upregulate the epidermal growth factor receptor through activation of mitogen-activated protein kinases and phosphatidylinositol-3-kinase in human ovarian surface epithelial cells. Endocr Relat Cancer 12: 407-421, 2005.
12. Choi JH, Choi KC, Auersperg N and Leung PC: Gonadotropins activate protein kinase A and phosphatidylinositol-3-kinase pathways in human epithelial ovarian cancer cells. Cancer Res 66: 3912-3920, 2006.
13. Zhao JX, Liu H, Lv J and Yang XJ: Wortmannin enhances cisplatin-induced apoptosis in human ovarian cancer cells in vitro. Eur Rev Med Pharmacol Sci 18: 2428-2434, 2014.
14. Kuroda H, Mandai M, Komishi I, Yura Y, Tsutaya Y, Hamid AA, Nunbu K, Matsuhashi K and Mori T: Human chorionic gonadotropin (hCG) inhibits cisplatin-induced apoptosis in ovarian cancer cells: possible role of up-regulation of insulin-like growth factor-1 by hCG. Int J Cancer 76: 571-578, 1998.
15. Zhang Z, Liao H, Chen X, Zheng Y, Liu Y, Tao X, Gu C, Dong L, Duan Y, Yang Y, et al: Luteinizing hormone upregulates survivin and inhibits apoptosis in ovarian epithelial tumors. Eur J Obstet Gynecol Reprod Biol 155: 69-74, 2011.
16. Yoon JH, Choi YJ, Kim SG, Nam SW, Lee JY and Park WS: Programmed cell death 6 (PDCD6) as a prognostic marker for gastric cancers. Tumour Biol 33: 485-494, 2012.
17. Park SH, Lee JH, Lee GB, Byun HJ, Kim BR, Park CY, Kim HB and Rho SB: PDCD6 additively cooperates with anti-cancer drugs through activation of NF-kB pathways. Cell Sign 24: 726-733, 2012.
18. Pon YL, Auersperg N and Wong AS: Gonadotropins regulate N-cadherin-mediated human ovarian surface epithelial cell survival at both post-translational and transcriptional levels through a cyclic AMP/protein kinase A pathway. J Biol Chem 280: 15438-15448, 2005.
19. Choi JH, Chen CL, Poon SL, Wang HS and Leung PC: Gonadotropin-stimulated epidermal growth factor receptor expression in human ovarian surface epithelial cells: Involvement of cyclic AMP-dependent exchange protein activated by cAMP pathway. Endocrinol Relat Cancer 16: 179-188, 2009.
20. Ohtani K, Sakamoto H, Kikuchi A, Nakayama Y, Idei T, Igarashi N, Matukawa T and Satoh K: Follicle-stimulating hormone promotes the growth of human epithelial ovarian cancer cells through the protein kinase C-mediated system. Cancer Lett 166: 207-213, 2001.
21. Huang Y, Hua K, Zhou X, Jin H, Chen X, Lu X, Yu Y, Zha X and Feng Y: Activation of the PI3K/AKT pathway mediates FSH-stimulated VEGF expression in ovarian serous cystadeno-carcinoma. Cell Res 18: 780-791, 2008.
22. Slot KA, de Boer-Brouwer M, Houweling M, Vaandrager AB, Slot H, van der Zee AGJ, de Vries EG and de Jong S: Drug-induced apoptosis in ovarian cancer cell line AS201. J Transl Med 10: 31, 2012.
23. Vito P, Lacanè E and D’Adamo L: Interfering with apoptosis: Ca(2+)-binding protein ALG-2 and Alzheimer’s disease gene ALG-3. Science 271: 521-525, 1996.