Gonadotropins Activate Proteolysis and Increase Invasion through Protein Kinase A and Phosphatidylinositol 3-Kinase Pathways in Human Epithelial Ovarian Cancer Cells

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Abstract

Despite evidence that gonadotropins may facilitate peritoneal metastasis of ovarian cancer by increasing cell adhesion, the action and molecular mechanism of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in ovarian cancer invasion is not well characterized. In the present study, we investigated the effects of FSH and LH on the invasive activity and the expression of metastasis-related proteases in human epithelial ovarian cancer by Western blot, zymography, reverse transcription-PCR (RT-PCR), ELISA, and Boyden chamber assay. Treatment with FSH or LH (10, 100, or 1,000 ng/mL) significantly increased the invasion of ovarian cancer cell lines, including BG-1, CaOV-3, and SKOV-3 cells but not OVCAR-3 cells. In addition, treatment of SKOV-3 cells with FSH or LH (100 or 1,000 ng/mL) enhanced the expression and activation of matrix metalloproteinases (MMP-2 and MMP-9) as shown by RT-PCR, gelatin zymography, and ELISA. Pretreatment with [(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-1-trotylan methylamide (10 μmol/L), a total MMP inhibitor, and 3-(4-phenoxypyphenylsulfonyl)-propylthiiranes (20 μmol/L), a specific gelatinase inhibitor, neutralized the proinvasive effect of gonadotropins in SKOV-3 cells. In addition, the secretion of tissue inhibitor of metalloproteinases (TIMP) for MMP and plasminogen activator inhibitor (PAI)-1 for uPA.

Introduction

Ovarian cancer is the sixth most common cancer and the fifth leading cause of cancer-related deaths among women in developed countries (1). Most deaths from ovarian cancer are due to metastases that are resistant to conventional therapies. Although ovarian cancers have been recognized to metastasize primarily by exfoliation followed by peritoneal implantation, ~40% of patient with advanced ovarian cancer show lymph node metastasis and/or extra-abdominal metastasis. However, the factors that regulate the metastatic process of ovarian cancer are poorly understood.

Recent findings suggested that proteolysis directed at the interface between ovarian cancer cells and peritoneal tissues may play a role in the localized invasion and dissemination of ovarian cancer cells in the peritoneal cavity (2, 3). Of various proteases, matrix metalloproteinases (MMP) and the urokinase-type plasminogen activator (uPA) system have been the most intensely investigated in ovarian cancer as well as in other cancers. The MMP family contains 24 human members, of which MMP-2 and MMP-9 (gelatinase A, 72-kDa type IV collagenase, and gelatinase B, 92-kDa type IV collagenase) have been observed in several ovarian cancer cell lines and detected in ascitic fluid from patients with advanced ovarian cancers. The invasiveness of ovarian cancer cells has been reported to correlate with the expression of MMP-2 and MMP-9 (4, 5). In ovarian cancer, uPA is also present in significant levels in ascites and increased levels are related to poor prognosis (6, 7). Furthermore, uPA and its receptor have been shown to increase in ovarian cancer cells (8). Both MMPs and uPA are secreted from the cells in a proform and become active through proteolytic cleavage. Once activated, the proteases can degrade the extracellular matrix and their activity is counterbalanced by their specific endogenous inhibitor, such as tissue inhibitor of metalloproteinase (TIMP) for MMP and plasminogen activator inhibitor (PAI)-1 for uPA.

Ovarian cancer is more common in conditions with elevated gonadotropins, such as postmenopausal women or women who have received treatment for induction of ovulation (9, 10). Additionally, reduced risk for ovarian cancer is associated with multiple pregnancies, breast-feeding, oral contraceptives, and estrogen replacement therapy, which are associated with lower levels and reduced exposure to gonadotropins (11, 12). The receptors for follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have been shown in normal and neoplastic ovarian surface epithelium (OSE) cells (13, 14). Moreover, levels of ovarian and peritoneal gonadotropins seem to be elevated in ovarian cancer patients (15, 16). Together, these observations suggest a possible role of gonadotropins in the development and progression of ovarian cancer. Although the mitogenic effect of gonadotropins is still controversial, their proliferative effect has been shown in OSE and ovarian cancer in vitro (14, 17–19). However, little is known about the effects of gonadotropins on other aspects of ovarian cancer, such as metastasis. Considering that gonadotropins may modulate invasiveness and interact with the MMP system in other cell models (20–23), we hypothesized that gonadotropins may play a role in ovarian cancer invasion via the MMP system. We did the present study to examine (a) the effect of gonadotropins on invasiveness of ovarian cancer cells, (b) the effect of gonadotropins...
on metastasis-related proteases, and (c) the involvement of protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K) signaling pathways in the regulation of invasiveness by gonadotropins.

Materials and Methods

Materials. Human LH and recombinant FSH were provided by Dr. A.F. Parlow (National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, Torrance, CA). 2-(Amino-3-methoxyphenyl)-4H-1-benzoazepin-4-one (PD98059), a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) inhibitor, was purchased from New England Biolabs, Inc. (Beverly, MA). 2-(4-Morpholinyl)-8-phenyl-1H-benzoazepin-4-one hydrochloride (LY294002), a specific cell permeable PI3K inhibitor, and [2-([p-bromocinnamylamino]ethyl)-5-isooquinolinesulfonyl] dihydrochloride (H89), a PKA inhibitor, were obtained from Sigma-Aldrich Corp. (St. Louis, MO). [(2R)-2-(hydroxy-amido-carbonylmethyl)-4-methylpentanoyl]-i-tryptophan methylethyl (GM6001), a broad-spectrum MMP inhibitor, and 3-(4-phenoxysulfon-fyl)-propylthiirane (SB-3CT), a selective MMP-2/MMP-9 inhibitor, were obtained from Chemicon (Temecula, CA). 4-(3-Choloroanilino)-6-dimethox-yquinazoline (AG1478), an epidermal growth factor (EGF) receptor (EGFR) kinase inhibitor, was purchased from Calbiochem (San Diego, CA).

Cell culture and treatment. OVCAR-3, CaOV-3, and SKOV-3 cells, ovarian cancer cell lines, were purchased from the American Type Culture Collection (Manassas, VA) and cultured as described previously (18) in Medium 199:MCDB 105 (1:1; Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5% CO2, 95% air at 37 °C. The cells were passaged using 0.06% trypsin (1:250) and 0.01% EDTA in Mg2+/Ca2+-free HBSS at (106 in inhibitor), SB-3CT (20 μL; a specific gelatinase inhibitor), GM6001, a broad-spectrum MMP inhibitor, and 3-(4-phenoxyphenylsulfonyl)-propylthiirane (SB-3CT), a selective MMP-2/MMP-9 inhibitor, were purchased from Calbiochem (San Diego, CA).

Invasion assay. In vitro cellular invasion was assayed by determining the ability of cells to invade a synthetic basement membrane (Matrigel, BD Biosciences, Mississauga, Ontario, Canada). Briefly, polycarbonate filters (8-μm pore size) were coated with Matrigel at a concentration of 1 μg/ml, and placed in a modified Boyden chamber. Trypsinized cells (1.5 x 105) were resuspended in Medium 199:MCDB 105 containing 0.5% FBS and various concentrations of FSH or LH and added to the top chamber in the presence or absence of pretreatment with GM6001 (10 μmol/L; a total MMP inhibitor), SB-3CT (20 μmol/L; a specific gelatinase inhibitor), LY294002 (10 μmol/L; a PI3K pathway inhibitor), or H89 (10 μmol/L; a PKA pathway inhibitor) for 20 minutes. Culture medium containing 1% FBS was then added to the bottom chamber. The cells were incubated at 37 °C and allowed to invade through the Matrigel barrier for 48 hours. Following incubation, filters were fixed and stained with crystal violet. Noninvading cells were removed using a cotton swab, whereas invading cells on the underside of the filter were counted using an inverted microscope. All experiments were done in triplicate, and a minimum of 10 fields per filter was counted.

Real-time PCR. Total RNA was prepared using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s instructions. Total RNA (2.5 μg) was reverse transcribed into first-strand cDNA (Amersham Pharmacia Biotech, Oakville, Ontario, Canada) following the manufacturer’s procedure. Briefly, the RNA solution was incubated at 65°C for 10 minutes and then chilled on ice. The bulk first-strand cDNA reaction mix (5 μL) 1 μL of 200 μmol/L DTT, 1 μL of 0.2 μmol/L, RoT-d (d)T16 primer, and the heat-denatured DNA were mixed and incubated at 37°C for 1 hour. The primers used for SYBR Green Real-time RT-PCR were designed using the Primer Express Software v2.0 (Perkin-Elmer Applied Biosystems, Foster City, CA) and were as follows: MMP-2, 5′-CCCGAGTGGAGCAAA-GATGT-3′ and 5′-CATTGGCGCTGTACATGGA-3′; MMP-9, 5′-GAG-TGCTCGACAGGT-5′ and 5′-CAATACAGCTGTTCACATC-3′; TIMP-1, 5′-ACAGTGCCGCCCCCTTTGGA-3′ and 5′-CAGCCCAACGACAACAGGAT-3′; TIMP-2, 5′-AGCATGACGACAGCTGGA-3′ and 5′-CCAAGGGAAA-GACCTGAAGGA-3′; uPA, 5′-CGCTTCTTCGTGGTTGCTA-3′ and 5′-CCAGCTCTCCTTTACGCTTATG-3′; PAI-1, 5′-CGCCGCGGCTCTTCACCA-3′ and 5′-GCACTGATGGGCACAGGAGA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATGAGAATCTTCACACCATCTT-3′ and 5′-GCCCAACTGGTATTGGG-3′. These primers were specific for MMP-2, MMP-9, TIMP-1, TIMP-2, uPA, PAI-1, and GAPDH as shown using the BLAST program (http://www.ncbi.nlm.nih.gov) and were purchased from Invitrogen. Real-time PCR was done using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems) equipped with a 96-well optical reaction plate. The reactions were set up with 12.5 μL SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems), 7.5 μL primer mixture (300 nmol/L) and 5 μL cDNA template. Real-time PCR conditions were as follows: 52 °C for 2 minutes followed by 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All real-time experiments were run in triplicate, and a mean value was used for the determination of mRNA levels. A negative control containing water instead of sample cDNA was used in each real-time plate. At the end of the PCR, baseline threshold values for these genes were set using the ABI 7000 Prism Software and the calculated Ct values were exported to Microsoft Excel for analysis. The relative expression of mRNA was calculated using the comparative Ct method according to manufacturer’s literature (Perkin-Elmer Applied Biosystems). The steady-state concentra-

Antibodies. MMP-2, MMP-9, and TIMP-1 antibodies were purchased from Neomarkers (Fremont, CA) and TIMP-2, uPA, and PAI-1 antibodies were acquired from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA). Phospho-AKT (Ser473), glycogen synthase kinase-3 (GSK3) α/β, forkhead in rhabdomyosarcoma (FKHR), pan-AKT, phospho–cyclic AMP (cAMP)–responsive element binding protein (CREB), and pan-CREB antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Immunoblot assay. Conditioned medium (30 μL) was loaded and transferred under reducing conditions on an 8% SDS-polyacrylamide gel to determine the secretion of MMPs, TIMPs, uPA, and PAI-1 from SKOV-3 cells. To examine the activation of PI3K and PKA signaling pathway, the cells were washed once with medium and serum was starved for 4 hours before treatments with FSH or LH (100 or 1,000 ng/mL) for 30 minutes. The cells were lysed in ice-cold radiomunoprecipitation assay buffer (150 μmol/mL NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 μmol/mL Tres (pH 7.5), 1 mmol/mL phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 100 μg/mL aprogin). Total protein (30 μg) was run on 10% SDS-polyacrylamide gels. After electrophoretic transfer, the proteins to a nitrocellulose membrane (Amersham Pharmacia Biotech), the membrane was immunoblotted using specific primary antibodies at 4 °C overnight. The signals were detected with horseradish peroxidase–conjugated secondary antibody for 1 hour and visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Zymography and reverse zymography. To measure the activity of MMPs, the conditioned medium was incubated with nonreducing dilution buffer before electrophoresis on an 8% SDS-polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, the gel was washed twice in 2.5% Triton X-100 to remove the SDS followed by incubation overnight at 37°C in buffer [0.1 mmol/L glycine, 10 mmol/L CaCl2, 1 μmol/L ZnCl2 (pH 8.3)] that allows both pro-gelatinase and active gelatinase to digest the gelatin. The gel was then stained with Coomassie blue G-250 (Bio-Rad Laboratories, Mississauga, Ontario, Canada) to visualize gelatinolytic activity. TIMP
activity by reverse zymography was assayed in polyacrylamide gels containing gelatin as a substrate copolymerized with recombinant pro-MMPs as described previously (24). Aliquots of conditioned medium were treated as described above for zymography, with the exception that the samples were separated on 15% polyacrylamide gels containing 0.1% SDS, 2.5 mg/ml gelatin, and 160 ng/ml recombinant pro-MMP-2.

ELISA. MMP-2, MMP-9, and TIMP-1 activity in conditioned medium was determined by using the Biotrak Assay (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Before ELISA analysis, samples were centrifuged at 14,000 rpm for 1 minute. Three ELISA experiments were conducted, with each sample done in duplicate. The absorbance of the samples was measured at 450 nm using a microplate spectrophotometer. A standard curve was generated from which the concentrations of MMP-2, MMP-9, and TIMP-1 were obtained.

Data analysis. Data are means ± SD of three individual experiments done in duplicate. For invasion assay, values are expressed as the percentage of invasion compared with control and are presented as mean ± SD of three individual experiments done in duplicate or triplicate. Data were analyzed by one-way ANOVA followed by Dunnett’s test, and P < 0.05 was considered statistically significant. Representative images of Western blot, zymography, and reverse zymography are shown.

Results

Effect of FSH and LH on ovarian cancer invasion. Numerous studies have shown the expression of FSH receptor (FSHR) and LH receptor (LHR) in ovarian cancer cells (13, 14). We reported previously that FSHR mRNA and protein are expressed in SKOV-3 and OVCAR-3 cells (25), and the expression of FSHR and LHR in the four ovarian cancer cells tested were confirmed by RT-PCR and Western blot analysis (data not shown). To examine the effect of gonadotropins on invasive capacity of ovarian cancer cells, the Boyden chamber assay using a Matrigel-coated invasion chamber was done. Although no significant change was observed in invasion of OVCAR-3 cells, which have little invasiveness in our cell culture system, we observed a significant increase in invasion of CaOV-3, BG-1, and SKOV-3 cells following the treatment with FSH or LH for 2 days (Fig. 1). FSH stimulated invasive activity in a dose-dependent manner (10, 100, and 1,000 ng/ml) with maximal 2- to 3-fold increase in CaOV-3, BG-1, and SKOV-3 cells. Although BG-1 and CaOV-3 cells showed significant response to only higher dose of FSH (100 and 100 ng/ml), 10 ng/ml FSH significantly increased invasive activity in SKOV-3 cells. In contrast, maximal 1.5-, 2.5-, and 2.2-fold increases were observed at 1,000 ng/ml LH in CaOV-3, BG-1, and SKOV-3 cells, respectively, and 100 ng/ml LH also showed a similar level of proinvasive effect. Considering that FSH and LH showed the most potent effect on invasion in SKOV-3 cells, additional experiments were done using this cell line to evaluate the mechanism of action of gonadotropins on the stimulation of invasion. In addition, using identical culture conditions as for invasion assay, we found that treatment of cells with gonadotropins did not induce any cell growth or cytotoxicity (data not shown).

Effect of gonadotropins on mRNA expression, secretion, and activation of MMPs and TIMPs in SKOV-3 cells. We examined whether gonadotropins modulate the secretion of MMP-2 and MMP-9 from SKOV-3 cells by Western blot analysis. As shown in Fig. 2A, 2-day treatment with FSH and LH resulted in enhanced secretion of MMP-2 and MMP-9, which was not affected by pretreatment with AG1478, an EGFR kinase inhibitor. To elucidate whether gonadotropin-induced MMP-2 and MMP-9 secretion was transcriptionally controlled, mRNA expression levels of MMP-2 and MMP-9 were examined by real-time PCR following treatment of SKOV-3 cells with FSH or LH. Dissociation curve analysis of MMP-2 and MMP-9 yielded a single peak, and their electrophoresis showed a single specific signal. Treatment with FSH or LH for 24 hours resulted in a significant up-regulation of MMP-2 and MMP-9 mRNA in SKOV-3 cells. Moreover, the treatment of the cells with actinomycin D, an inhibitor of transcription, abolished the effects of gonadotropins on MMP-2 and TIMP expression (Fig. 2B), suggesting that enhanced MMP secretion by gonadotropins may be regulated at the transcriptional level. Because active MMP-2 and MMP-9 could not be detected by Western blotting, more sensitive zymography and ELISA kits were used to measure the activity of MMP-2 and MMP-9 following treatment of SKOV-3 cells with FSH or LH. Because the gels were
stained with Coomassie blue to visualize the enzymatic digestion, the lysis zones representing the enzymatic digestion appeared as clear zones in the zymography gel. As shown in Fig. 2C, treatment with FSH and LH increased the active forms of MMP-2 (66 kDa) and MMP-9 (86 kDa) as well as their latent forms (72 and 92 kDa, respectively) in a dose-dependent manner (100 and 1,000 ng/mL) in SKOV-3 cells. The lytic zones at 66, 72, 88, and 92 kDa were easily inhibited by incubation of the gel with EDTA, a calcium chelator, thus confirming that the activity bands represent MMPs. These results were confirmed by an ELISA assay specific for the active forms of MMP-2 and MMP-9 (Fig. 2D). In addition, the secretion pattern of pro-MMPs in zymography was similar to that observed by immunoblot analysis data. TIMPs, the endogenous inhibitors of MMPs, can be regulated by various factors in human cancers and bound to the catalytic site of MMPs in 1:1 ratio. In this study, mRNA expression and secretion of TIMP-1 and TIMP-2 were markedly decreased by treatment with gonadotropins in a dose-dependent manner as shown by immunoblot and ELISA assays (Fig. 3A-C). We
showed that decreased secretion of TIMPs resulted in a reduction in the inhibition of MMPs as assessed by reverse zymography, in which dark bands were undigested gelatin stained with Coomassie blue representing areas of MMP inhibition (Fig. 3D).

**Effect of gonadotropins on mRNA expression, secretion, and activation of uPA and PAI-1 in SKOV-3 cells.** Because uPA was also found at high concentration and its level correlated inversely with prognosis in ovarian ascites and ovarian cancers (8), we investigated whether gonadotropins could stimulate uPA mRNA expression and its release in SKOV-3 cells using real-time PCR and immunoblot analysis. Neither mRNA nor protein level of uPA was altered; whereas expression level of PAI-1, an endogenous inhibitor of uPA, was moderately decreased by treatment with FSH or LH (Fig. 4).

**Inhibitory effect of MMP inhibitors on gonadotropin-induced invasion in SKOV-3 cells.** To determine the role of these proteases in the gonadotropin-dependent invasion in SKOV-3 cells, a broad-spectrum MMP inhibitor (GM6001) and a specific gelatinase (MMP-2 and MMP-9) inhibitor (SB-3CT) were used. At the concentration used in our studies and others, these MMP inhibitors have no inhibitory effect on basal invasion rate in ovarian cancer (26). The gonadotropin-induced increase in invasion was significantly reduced by the addition of 10 μmol/L GM6001 and 20 μmol/L SB-3CT as shown in Fig. 5. It is of interest that the inhibitory effects of SB-3CT on gonadotropin-induced invasion were similar to those of GM6001, suggesting that only gelatinases, including MMP-2 and MMP-9 and not other groups of MMPs, play an essential role in ovarian cancer invasion stimulated by gonadotropins.

**Involvement of PKA and PI3K pathway in gonadotropin-induced invasion and MMP secretion in SKOV-3 cells.** To address the signaling pathway involved in the proinvasive effect of gonadotropins, we examined the effects of specific signaling inhibitors on gonadotropin-induced invasion. We found that H89, a PKA inhibitor, and LY294002, a PI3K inhibitor, markedly blocked gonadotropin-induced invasion, whereas PD98059 (an ERK-1/ERK-2 kinase inhibitor), GF109203X [a protein kinase C (PKC) inhibitor], and SB203580 (a p38 inhibitor) did not cause a substantial inhibition (Fig. 6d). Moreover, H89 and LY294002 significantly inhibited the gonadotropin-induced MMP-2/MMP-9 secretion (Fig. 6B). These results indicate that gonadotropins may increase SKOV-3 cell invasion by activating the PKA and PI3K signaling pathways. To further test the effect of FSH and LH on activation of the PKA and PI3K signaling cascades, we examined the phosphorylation status of their downstream second messengers, such as CREB and Akt, after treatments of the cells with FSH and LH (100 and 1,000 ng/mL) for 30 minutes. We did an immunoblot analysis with specific antibodies to detect phosphorylated forms of Akt, GSK3α/β, FKHR, and CREB, and total Akt and CREB for normalization. FSH and LH induced phosphorylation of Akt (Ser473) and CREB at 30 minutes (Fig. 6C and D). The activation of the PI3K pathway was confirmed by the increased phosphorylation of the downstream proteins, GSK3α/β and FKHR (Fig. 6C). FSH- and LH-induced activations of Akt, GSK3α/β, and FKHR were

![Figure 3. Effect of gonadotropins on mRNA expression, secretion, and activation of TIMP-1 and TIMP-2 in SKOV-3 cells. A, conditioned medium was collected from SKOV-3 cells after 2-day incubation with FSH or LH (100 and 1,000 ng/mL). The supernatants were normalized based on viable cell number (MTT assay) and β-actin protein levels. Western blot was done for TIMP-1 and TIMP-2 (28 and 21 kDa) as described in Materials and Methods. B, following 1-day incubation with FSH or LH (100 and 1,000 ng/mL), expression of mRNA transcripts for TIMP-1 and TIMP-2 was analyzed by real-time RT-PCR. C, TIMP-1 secretion was measured in conditioned medium from SKOV-3 cells treated with FSH or LH (100 ng/mL) for 1 or 2 days using an ELISA assay kit. D, enzymatic activity of TIMP-1 and TIMP-2 was studied in conditioned medium from SKOV-3 cells by SDS-PAGE reverse zymography for TIMP-1 and TIMP-2 (28 and 21 kDa). Columns, mean of three individual experiments; bars, SD. a, P < 0.05 versus nontreated cells; b, P < 0.05 versus 100 ng/mL FSH- and LH-treated cells.](cancerres.aacrjournals.org/dist/fig/3.png)
completely abolished by pretreatment with LY294002, a PI3K inhibitor. Similarly, the PKA inhibitor H89 completely abolished FSH- and LH-induced phosphorylation of CREB. Treatment with these inhibitors did not result in any cytotoxic effects under the present experimental condition (data not shown). These results suggest that FSH and LH activated the PI3K and PKA pathways in SKOV-3 cells, which may play an important role in invasion of ovarian cancer.

Discussion

Because the hypothesis that pituitary gonadotropins increase the risk of ovarian malignancy and that pregnancies and oral contraceptives protect the ovary by suppressing secretion of these hormones was suggested (27), numerous studies have examined the role of gonadotropins on ovarian cancer. Treatment with FSH and LH/human chorionic gonadotropin (HCG) seemed to stimulate the growth of normal and immortalized OSE (IOSE) and several ovarian cancer cell lines in a dose- and time-dependent manner in vitro. Although these observations have shown the role of gonadotropins in initiation and proliferation of ovarian cancer, the contribution of gonadotropins to other aspects of cancer progression, such as metastasis, is poorly understood. Gonadotropins have been reported to enhance tumor angiogenesis and adhesion by regulating the expression of vascular endothelial growth factor and integrin subunit α(v) and CD44 in ovarian cancer cells (20, 28, 29). In addition, LH/HCG seems to regulate cell-matrix adhesion and proteolysis during physiologic or pathologic processes, such as trophoblast invasion, ovulation, and metastasis of breast and endometrial cancer (21, 22, 30). Thus, we did the present study to test the hypothesis that gonadotropins may affect ovarian cancer metastasis by regulating invasion and/or proteolysis.

The effect of gonadotropins on ovarian cancer cells using an invasion chamber coated with Matrigel was primarily examined. Treatment with gonadotropins significantly enhanced the invasiveness of three ovarian cancer cell lines, BG-1, CaOV-3, and SKOV-3, whereas no change was observed in OVCAR-3 cells. We cannot explain why gonadotropins have no effect on OVCAR-3 cells among the four ovarian cancer cell lines tested in this study. It can be assumed that distinct effects may be proportional to the innate invasive capacity of these cell lines, as the proinvasive effect of gonadotropins was greatest in SKOV-3 cells (most invasive cells in our culture system), whereas no effect was observed in OVCAR-3 cells (least invasive cells). It is commonly believed that EGFR expression is correlated with potent invasiveness as well as poor prognosis in ovarian cancer, and we showed previously that the more invasive SKOV-3 cells have higher EGFR levels than OVCAR-3 cells. In addition, EGF stimulated MMP-9 production by regulating PI3K signaling in ovarian cancer (31). In this regard, the possibility exists that EGFR could somehow be involved in gonadotropin-stimulated invasion. We showed previously that treatment with gonadotropins increased EGFR in IOSE and OVCAR-3 cells but not in SKOV-3 cells (32). In this study, we found that AG1478, EGFR kinase inhibitor, did not show any significant inhibition of gonadotropin-induced MMP-2/MMP-9 secretion in SKOV-3 cells (Fig. 2D) and gonadotropin-induced PI3K activation. Moreover, the phosphorylation of EGFR was not affected by gonadotropin treatment. These data suggest that the regulation of EGFR expression or activation does not seem to be the primary mechanism for, at least, gonadotropin-induced invasion in SKOV-3 cells.

There is evidence that the expression and activation of MMPs and uPA are regulated by various cellular factors (EGF, transforming growth factor, lysophosphatidic acid, and endothelin) and...
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Experiments; PD98059 (\textsc{a}) the cells were treated with FSH or LH (100 ng/mL) and the invasion assay (\textsc{b}) was done. Immunoblot analysis was done with increasing doses of FSH and LH (100 and 1,000 ng/mL) for 30 minutes. \textsc{c}, phosphorylated AKT (\textsc{p-Akt}; Ser473), GSK3 (\textsc{p-GSK3}), and FKHR (\textsc{p-FKHR}) levels were normalized by total AKT (\textsc{T-Akt}). \textsc{d}, phosphorylated CREB (\textsc{p-CREB}) levels were normalized by total CREB (\textsc{T-CREB}). Columns, mean of three individual experiments; bars, SD. \textsc{a}, \textit{P} < 0.05 versus nontreated cells.

Figure 6. Involvement of PI3K and PKA signaling pathways in gonadotropin-induced invasion and MMP production of SKOV-3 cells. Following 20-minute pretreatment with 10 μmol/L LY294002 (+LY; PI3K inhibitor), 10 μmol/L PD98059 (+PD; MAPK inhibitor), 10 μmol/L GF109203 (+GF; PKA inhibitor), 20 μmol/L SB203580 (+SB; p38 inhibitor), or 10 μmol/L H89 (+H89; PKA inhibitor), the cells were treated with FSH or LH (100 ng/mL) and the invasion assay (\textsc{a}) was done. Immunoblot analysis was done with increasing doses of FSH and LH (100 and 1,000 ng/mL) for 30 minutes. \textsc{c}, phosphorylated AKT (\textsc{p-Akt}; Ser473), GSK3 (\textsc{p-GSK3}), and FKHR (\textsc{p-FKHR}) levels were normalized by total AKT (\textsc{T-Akt}). \textsc{d}, phosphorylated CREB (\textsc{p-CREB}) levels were normalized by total CREB (\textsc{T-CREB}). Columns, mean of three individual experiments; bars, SD. \textsc{a}, \textit{P} < 0.05 versus nontreated cells.

The actions of FSH and LH on their principal target cells, such as granulosa and theca cells in the ovary, are mediated primarily by their G-protein-coupled transmembrane receptors. Furthermore, substantial data support the idea that gonadotropins induce cAMP production and activate the PKA pathway when they bind to their receptor. In addition, gonadotropins may cause an activation of PI3K and AKT in other reproductive tissue, including granulosa cells, Sertoli cells, and oocytes (38–40). Gonadotropins have been also shown to activate ERK and p38 MAPK in granulosa cells (41, 42). As for ovarian cancers, we showed previously that both FSH and LH increased EGFR levels through activation of MAPK and PI3K in human OSE cells (32). Indeed, FSH-induced activation of the MAPK cascade and phosphorylated Elk-1 is responsible for its effects on proliferation in OSE and ovarian cancer cells (18). In addition, Ohtani et al. (19) found that FSH significantly up-regulated the levels of PKCα mRNA and protein and proposed the involvement of the PKC pathway in FSH-induced cell proliferation in ovarian cancer cells. FSH and LH also stimulated the growth of human OSE and ovarian cancer cells through the PKA and PI3K and PKA signaling pathways. Aberrant activity of the PI3K signaling pathway in human cancer is of great interest. In ovarian cancer, the PI3K signaling pathway plays a role in proliferation, antiapoptosis, and tumorigenesis (44). Moreover, increasing evidence suggests the involvement of PI3K in cell migration, invasion, and metastasis in normal and neoplastic tissues, including ovarian cancer (45). Up-regulation of AKT2 stimulated invasion by overexpression of integrin-β1 (46). PKA, a serine/threonine kinase, is the main mediator of cAMP signaling in mammals. Although several studies that the regulation of proteases by these factors eventually affect invasion and metastasis (2, 8, 26, 33). Gonadotropins are also involved in the activation and/or expression of MMP and TIMP family members in the physiology and pathophysiology of the ovary and trophoblast (21–23). These observations prompted us to investigate whether gonadotropins may regulate these tumor protease systems. Both FSH and LH increased the expression and activation of MMP-2 and MMP-9 and decreased the expression and activation of their inhibitors, TIMP-1 and TIMP-2, in SKOV-3 cells, indicating that gonadotropins enhance the net MMP/TIMP ratio and the capacity for proteolysis in ovarian cancer. Because an incomplete inhibition of invasion implies other mechanisms, we further evaluated the involvement of the uPA system, which is also essential for invasion. Treatment of SKOV-3 cells with FSH and LH did not result in any significant change in uPA mRNA expression and secretion. On the other hand, its inhibitor PAI-1 was moderately decreased by gonadotropins. This is in agreement with a previous study showing that secretion of uPA was unaffected by human menopausal gonadotropins in SKOV-3 cells (34). The fact that PAI-1 reduced the migration and invasion of SKOV-3 cells due to its ability to inhibit uPA activation (35) supports the possibility that down-regulation of PAI-1 by gonadotropins contributes to the stimulation of ovarian cancer invasion. Other studies suggest that PAI-1 not only inhibits uPA but also functions in cell adhesion, angiogenesis, and apoptosis to contribute to increased metastasis (36, 37). As for a partial inhibitory effect of MMP inhibitors on gonadotropin-induced invasion, one possible explanation would be that gonadotropins may play a role in other steps of invasion, such as cell-matrix adhesion as well as proteolysis. We did not test the involvement of adhesion mechanisms in the proinvasive effect of FSH and LH in this study. However, it is of interest that treatment of the epithelial ovarian carcinoma MLS with gonadotropins up-regulated CD44 and α(β)-integrin expression and consequently augmented cell adhesion (20).
have shown a role of PKA in suppressing the growth of different types of cancers, including ovarian cancer cells (47). PKA has been recently reported to positively regulate cell migration and invasion by increasing cell-substrate adhesion and/or MMP activation in different cellular models (21, 48, 49).

Both FSH and LH, which have dissimilar roles in granulosa and theca cells, respectively, have proinvasive activity on ovarian cancer in this study. The parallel effect of gonadotropins on cell growth, adhesion, and the expression of growth factors and their receptors as well as coexpression of gonadotropin receptors in various normal OSE and ovarian cancer models were shown (14, 17, 20, 29, 32, 43). Considering that FSHR and LHR share various signaling molecules, a parallel effect of gonadotropins on cell growth, adhesion, and the expression of growth factors and their receptors and as well as coexpression of gonadotropin receptors in normal OSE and ovarian cancer is promising. For example, PKA-mediated STAT3 activation was involved in both FSH- and LH-induced proliferation in IOSE and ovarian cancer cells (43). These results were different from those of Ivanov et al. (50), which showed antiproliferative effects of FSH on OSE and the absence of effect by LH. In contrast, Zheng et al. (13) showed ovarian cancer growth stimulation by FSH and inhibition of the effect by LH. The cause(s) of the discrepancies between the reports remains to be determined. Taken together, our results indicate that gonadotropins may increase proteolysis-dependent invasion by activating the PI3K and PKA pathways. Because ovarian cancer progression involves metastasis and is more common in high levels of gonadotropins, understanding the role of gonadotropins in invasion and/or metastasis on cellular and molecular levels may help elucidate the etiology of ovarian cancer development.

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