Reproductive Hormone-Induced, STAT3-Mediated Interleukin 6 Action in Normal and Malignant Human Ovarian Surface Epithelial Cells

Vigar Syed, Gregory Ulinski, Samuel C. Mok, Shuk-Mei Ho

Background: Reproductive hormones are associated with risk for epithelial ovarian cancer. To determine the effect of such hormones on the activation of interleukin 6 (IL-6)/STAT3 (signal transducer and activator of transcription-3) signaling, which may be involved in ovarian cancer, we investigated the status of STAT3, IL-6, and its receptor in immortalized human ovarian surface epithelial (HOSE) and ovarian cancer (OVCA) cell lines. Methods: Two immortalized HOSE cell lines and two OVCA cell lines were cultured with gonadotropins, sex steroid hormones, and/or IL-6, alone or with specific inhibitors or IL-6-neutralizing antibodies. Expression of IL-6, the IL-6 receptor α chain (IL-6Rα), and phosphorylated and unphosphorylated STAT3 messenger RNAs (mRNAs) and proteins in all cells was determined. Cell proliferation and soft-agar colony formation were assessed. STAT3 activity was investigated in OVCA cells transfected with a dominant negative STAT3 (Dn-STAT3), wild-type STAT3, or an empty control vector. All statistical tests were two-sided. Results: Levels of IL-6 mRNA and protein increased in all cells treated with follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17β-estradiol, or estrone but increased only in OVCA cells treated with testosterone and 5α-dihydrotestosterone. For all lines, IL-6 antibodies partially inhibited hormone-stimulated cell proliferation but completely inhibited IL-6-enhanced cell proliferation. IL-6 induced STAT3 phosphorylation and activation in HOSE cells; STAT3 was constitutively activated in OVCA cells. Higher levels of IL-6Rα and STAT3 transcription factors were observed in OVCA cells than in HOSE cells. After transfection, Dn-STAT3 suppressed endogenous STAT3 and inhibited all forms of IL-6-stimulated OVCA cell proliferation (OVCA 429 cells, P<.001; OVCA 432 cells, P<.006), whereas wild-type STAT3 enhanced HOSE cell proliferation (wild-type STAT3 at 0.5 µg/mL in HOSE 306 cells, P<.002; STAT3 at 1.0 µg/mL in HOSE 306 or both concentrations of wild-type STAT3 in HOSE 642 cells, P<.001). Conclusions: The IL-6/STAT3 signaling pathway may mediate FSH-, LH-, and estrogen-stimulated HOSE cell proliferation. Increased IL-6Rα expression and constitutive STAT3 activation may be associated with ovarian cancer. [J Natl Cancer Inst 2002;94:617–29]
patients with ovarian cancer (41,42). OVCA cell lines NOM1 and SKOV cultured with IL-6 have increased chemotactic and/or chemokinetic activity and increased overall invasiveness (43). Thus, IL-6 appears to be an important regulator of OVCA cell growth and/or survival. However, to the best of our knowledge, no information is available on 1) the action of IL-6 on normal HOSE cell function, 2) the upstream regulators of IL-6 expression in HOSE cells and OVCA cells, and 3) the characteristics of the IL-6/STAT3 signaling cascade of OVCA cells.

It is well documented that, apart from gonadotropins and ovarian steroids, the ovary is regulated by many cytokines, including IL-6, that play important roles in folliculogenesis and oocyte maturation. The effects of exogenous gonadotropins on the release of IL-6 into the human follicular fluid have been studied (44). Gonadotropins have been shown to regulate ovarian secretion of IL-6 because IL-6 levels in follicular fluid were elevated after gonadotropin treatment (44,45). Gonadotropins have been shown to stimulate IL-6 production in Sertoli cells (46). The effect of androgen on the expression of IL-6 varies. In stromal cells derived from murine bone marrow (47), brain glial cells and astrocytes (48), and a human osteoblastic cell line (49), androgens inhibit IL-6 production. However, in Sertoli cells, low doses of testosterone stimulate IL-6 expression but higher doses inhibit IL-6 expression (46). Estrogens have been reported to enhance IL-6 expression in blood mononuclear cells in vitro (50) but to inhibit IL-6 synthesis in a human osteoblastic cell line, vascular smooth muscle cells, and bone marrow-derived stromal cells (51–53).

Regulation of IL-6 in HOSE cells has not been studied. We thus investigated whether gonadotropins, estrogens, and androgens affect IL-6 expression in HOSE and OVCA cells and whether IL-6 participates in the proliferation of HOSE and OVCA cells by activating STAT3. We also examined the level of IL-6Rα expression and the state of STAT3 activation in HOSE and OVCA cells.

**MATERIALS AND METHODS**

**Cell Cultures and Cell Lines**

The origin and culture conditions of HOSE and OVCA cell lines have been previously described (12,17,54). In short, HOSE cell lines HOSE 301, HOSE 306, HOSE 642, and HOSE 12–12 were derived from normal ovaries obtained from women with noncancerous gynecologic indications—specifically, a 47-year-old woman with endometrial adenocarcinoma, a 53-year-old woman with breast cancer, a 47-year-old normal woman, and a 39-year-old woman with ovarian stromal hyperplasia, respectively. Primary cultures were established from surface scrapings of these normal ovaries and immortalized with human papillomavirus E6 and E7 genes (55). All primary cultures had an epithelial cell phenotype, i.e., cobblestoned morphology, epithelial cytokeratin staining, responsiveness to transforming growth factor-β, no detectable CA125 production, and lack of in vivo tumorigenicity (55). OVCA cell lines OVCA 420, OVCA 429, OVCA 432, and OVCA 433 were established cell lines derived from patients with late-stage serous ovarian adenocarcinomas, as described by Bast et al. (56). These cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air in a 1 : 1 mixture of medium 199/MCDB 105 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma), penicillin (100 U/mL; Sigma), and streptomycin (100 µg/mL; Sigma).

**Hormone Treatment of HOSE and OVCA Cell Lines**

Cell lines cultured in medium 199/MCDB 105 were harvested when 80% confluent and washed twice in phosphate-buffered saline (PBS), and then 2 x 10^5 cells were cultured per T-25 flask (culture area = 25 cm²; Falcon; Becton Dickinson Labware, Bedford, MA) and allowed to attach for 24 hours. Forty-eight hours later, the medium was replaced with medium 199/MCDB 105 containing FBS treated with activated charcoal (Sigma)/dextran-70 (hereafter, charcoal-stripped FBS; Pharma- cia, Peapack, NJ), and 1 µM FSH (Calbiochem, San Diego, CA), 1 µM LH (Calbiochem), 1 µM 17β-estradiol (Sigma), 1 µM estrone (Sigma), 1 µM 5α-dehydrotestosterone (Sigma), or 1 µM testosterone (Sigma). Steroid solutions were in absolute ethanol, and gonadotropin solutions were in aqueous saline. The final concentration of ethanol in the medium was 0.1%. Untreated control cultures were exposed to equal concentrations of ethanol or aqueous saline vehicle alone. Cells were cultured for 5 days with or without hormones. Because 5α-dehydrotestosterone was rapidly metabolized, 5α-dehydrotestosterone was added every 12 hours.

**Hormone Treatment of Normal Immortalized and Malignant HOSE Cells in the Absence and Presence of Hormone Receptor Antagonists**

HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells (2 x 10^5 cells per T-25 flask) were allowed to attach for 24 hours, and then 10 nM FSH, 10 nM LH, 10 nM testosterone, or 10 nM 17β-estradiol was added in the presence or absence of the corresponding receptor or signaling inhibitor. A dose of 10 nM was chosen because it fell at the mid-point of dose–response curves from the cell proliferation assay. The protein kinase A-selective inhibitor H-89 (N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCL; Calbiochem) with an inhibition constant of 0.048 µM was added to a final concentration of 0.1 mM 30 minutes before treatment with 10 nM FSH or 10 nM LH. H-89 at 0.1 mM effectively blocks the action of FSH and LH (17). Receptor-specific inhibitors were used for sex steroid hormones as follows: 0.1 mM 4-hydroxyflutamide (Schering-Plough, Kenilworth, NJ), an androgen receptor inhibitor (17), and 0.1 mM ICI 182,780 (a gift from Zeneca Pharmaceuticals, Macclesfield, U.K.), a pure estrogen receptor inhibitor (17). Cell cultures were treated daily with hormones or hormone inhibitors for a period of 5 days, cells were collected, and total RNA was extracted. Each experiment was carried out twice.

**Treatment of HOSE and OVCA Cell Lines With Hormone and IL-6 Antibody**

HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were cultured in wells of 96-well plates at 1000 cells per well in medium containing charcoal-stripped FBS. Forty-eight hours later, the medium was replaced with the same medium containing human FSH, human LH, 5α-dehydrotestosterone, testosterone, 17β-estradiol, or estrone (each at 10 nM), as described above (17). Control cells were exposed to PBS or ethanol vehicle alone. To neutralize hormone-induced IL-6, cells were cultured for 5 days with hormones and a rabbit anti-human IL-6 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a final concentration of 50 µg/mL. The specificity of this anti-IL-6 antibody was confirmed by its complete inhibition of the mitogenic effect of recombinant IL-6 (100 ng/mL).
in the TF-1 cell line. To ensure stable availability, hormones and anti-IL-6 antibody were added to cells daily. Five days after treatment, cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit (Roche Diagnostics, Indianapolis, IN).

IL-6 Treatment of HOSE and OVCA Lines

Cells were cultured, as described above, in 96-well plates at 1000 cells per well in medium 199/MCDB 105 containing 10% charcoal-stripped FBS. Forty-eight hours later, human recombinant IL-6 (Upstate Biotechnology, Lake Placid, NY) at 1–32 ng/mL was added to wells, and the same concentration of IL-6 was added daily for 5 days. On day 6, the number of cells in each well was measured by use of an MTT cell proliferation assay (Roche Diagnostics). For one set of experiments, rabbit anti-human IL-6 polyclonal antibody (Santa Cruz Biotechnology) at 50 μg/mL was added to the cultures to neutralize IL-6, and 5 days later cell proliferation was assessed with the MTT assay. For other experiments, 2 × 10⁶ HOSE cells or 2 × 10⁵ OVCA cells per T-25 flask (Falcon) were allowed to attach for 24 hours. Forty-eight hours later, cells were treated with IL-6 at 32 ng/mL for 6 hours or 5 days, and then cell lysates were prepared.

Cell Growth Assay

The number of cells in each culture was measured with an MTT cell proliferation kit (Roche Diagnostics). The MTT labeling reagent (10 μL, final concentration = 0.5 mg/mL) was added to the medium in each culture well and incubated for 4 hours in a humidified atmosphere, 100 μL of a solubilization solution (10% sodium dodecyl sulfate in 0.01 M HCl) was added to each well, and plates were incubated overnight at 37 °C. The number of cells in each culture is linearly related to the conversion of the tetrazolium compound to the colored formazan product with an absorbance at 570 nm. Assays were performed in triplicate. The relative cell growth for each treatment regimen is expressed as the fold increase over that of untreated control cultures. The number of cells in the untreated control cultures was arbitrarily assigned a value of one, and values from treatment groups were normalized to values from the control cultures.

RNA Isolation and Quantification of IL-6, IL-6Rα, and gp130 Messenger RNA (mRNA) by a Semiquantitative Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

Total RNA was isolated from treated and untreated HOSE and OVCA cell lines with Tri reagent (Sigma) according to the manufacturer’s instructions and quantified by UV absorbance at 260–280 nm. The integrity of RNA was confirmed as previously described (54). Total RNA (1–3 μg) from each sample was reverse transcribed by use of a GeneAmp RNA PCR kit (PerkinElmer/Cetus, Norwalk, CT). The resulting complementary DNAs (cDNAs) were stored at −20 °C until use.

To investigate the relative expression of IL-6, IL-6Rα, and gp130 mRNA in each culture, semiquantitative RT–PCRs were performed. Sequences of the oligonucleotide primers specific for human IL-6, IL-6Rα, and gp130 were from published sequences (41). The forward primer sequence for IL-6 was 5′-CTTCGGTGCCATTGAGCTTTCT-3′ and the reverse primer was 5′-AGGA-ACCTCCTTAAAAGCTGCG-3′. For human IL-6Rα, the sense primer was 5′-GAGGAGACAGCTCTTCTTCTAC-3′ and the antisense primer was 5′-CCCTCAGCCCAGTATCTGAG-3′. For gp130, the sense primer was 5′-ACCTATGAGAGATGACCATCTAA-3′ and the antisense primer was 5′-GGTTTCTAA-AAATATGCTATAAT-3′. For amplification of human 18S ribosomal RNA (rRNA), the sense primer was 5′-TGAGGCCCATGTTAAGGGG-3′ and the antisense primer was 5′-CGCTGAGCCAGCTGTTGT-3′. These primers are expected to yield products of 609 base pairs (bp) for IL-6, 768 bp for IL-6Rα, 720 bp for gp130, and 623 bp for 18S rRNA. Aliquots of 1–3 μL of first-strand cDNAs were amplified by PCR. Hot-start PCR with AmpliTaq Gold DNA polymerase (PerkinElmer/ Cetus) was used to minimize the amplification of nonspecific products. The enzyme was activated by preheating to 95 °C for 6 minutes. Initially, to determine conditions for logarithmic-phase PCR amplification of IL-6, IL-6Rα, gp130, and 18S rRNA, various amounts of total RNA were reverse transcribed, and aliquots were amplified for various numbers of PCR cycles. A linear relationship was observed between the amount of RNA and PCR products when 3 μg of total RNA was used in the reverse transcription reaction and 32, 24, 28, and 18 PCR amplification cycles, respectively, were performed for IL-6, IL-6Rα, gp130, and 18S rRNA. The PCR program was 1 minute at 94 °C, 1 minute at 60 °C (annealing temperature), and 1 minute at 72 °C. mRNA-specific modifications included annealing temperatures of 58 °C for IL-6 cDNA, 58 °C for IL-6Rα cDNA, and 60 °C for gp130 cDNA. PCR products were fractionated on a 2% agarose gel and quantified after ethidium bromide staining and UV transillumination as follows. Images were captured with a digital camera (Kodak EDAS 290; Eastman Kodak, Rochester, NY) and converted to digitized signals. Intensities of each band were quantified by the Kodak 10 Image analysis software version 3.5. (Eastman Kodak). Signal intensities of target products (IL-6, IL-6Rα, and gp130) were normalized to that of the 18S rRNA product to obtain arbitrary units of relative message abundance. Reproducibility was evaluated by synthesis of three independent cDNAs and PCR runs from each RNA preparation. Means of the replicated measurements were calculated.

Immunoblot Analysis

To obtain cellular extracts, cells were lysed in lysis buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 0.5% Nonidet P-40), and proteins were quantified with a protein determination kit (Pierce, Rockford, IL). Proteins (25 μg) from HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels and transferred to nitrocellulose membranes. Blots were blocked with 5% bovine serum albumin or 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 for 1 hour. Blots were probed with anti-STAT3 antibody (1.5 μg/mL; Upstate Biotechnology), anti-phospho-STAT3 antibody (1:1000 dilution; Cell Signaling, Beverly, MA), anti-IL-6 antibody (1:600 dilution, Santa Cruz Biotechnology), anti-IL-6Rα antibody (1:500 dilution; Santa Cruz Biotechnology), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:500 dilution; Biogenesis, Poole, U.K.) overnight at 4 °C and then incubated with a secondary antibody (1:6000 dilution) in 3% bovine serum albumin or 3% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 for 1 hour at room temperature. After washing, bound antibodies were detected by modified chemiluminescence with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Bio...
Electrophoretic Mobility Shift Assays (EMSAs)

The sequence of the double-stranded Stat3 consensus oligonucleotide used as the probe in gel-shift experiments corresponded to the highest affinity binding STAT3 site (5′-GATCCCTCG-GGAATTCTAGATC-3′ and 3′-CTAGGAAGACGCTTAAG-GATCTAG-5′). This oligonucleotide is very specific for STAT3 and has been extensively used to identify STAT3 transcription factors (27). The Stat3 consensus oligonucleotide and the mutant STAT3 oligonucleotide, which contained an AAT-to-CCG substitution in the STAT3 binding motif, were purchased from Santa Cruz Biotechnology. Probes were labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Promega, Madison, WI).

Cells were homogenized in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.05% Nonidet P-40. Protease inhibitors ( aprotinin at 1 μg/mL, leupeptin at 1 μg/mL, pepstatin at 1 μg/mL) were added to minimize protein degradation. After incubation on ice for 15 minutes, the homogenate was centrifuged at 10,000 rpm in a microcentrifuge at 4 °C for 10 minutes. The pellet was resuspended in buffer containing 20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 6.25% glycerol. After incubation on ice for 40 minutes, the nuclear suspension was centrifuged at 15,000 rpm in a microcentrifuge at 4 °C for 10 minutes. The supernatant was collected and protein concentrations determined by the Bio-Rad protein assay. Nuclear extracts were incubated with 1 μg of poly(dI-dC) (Roche Diagnostics Corp., Indianapolis, IN) and labeled oligonucleotides for 25 minutes, and then the protein–DNA complexes were resolved by electrophoresis in a 6% nondenaturing polyacrylamide gel. Gels were dried and radioautographed at −70 °C.

To investigate the sequence specificity of the protein–DNA interaction, a 100-fold molar excess of unlabeled Stat3 oligonucleotides (specific competitor) or mutant STAT3 oligonucleotides (nonspecific competitor) was added to the binding reaction, and displacement of the radioactive probe was determined for both oligonucleotides.

Transient Transfection

To monitor transient transfection, HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells (1 × 10^5 cells) were transfected with the pSV-β-galactosidase control vector (Promega, Madison, WI) at 1 μg/mL with LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD). Cells transfected with the pSV-β-galactosidase control vector express β-galactosidase and thus can be identified and used to calculate transfection efficiency. Briefly, after transfection, cells were fixed in glutaraldehyde for 15 minutes, extensively washed in 1× PBS, and incubated with 0.2% X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) in PBS containing 2 mM MgCl2, 5 mM K4Fe(CN)6·3H2O, and 5 mM K3Fe(CN)6. After fixation and incubation with X-Gal, transfected cells contained a blue reaction product. Transfection efficiencies of HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells transfected with the pSV-β-galactosidase vector, estimated by X-Gal staining, were 71%, 68%, 75%, and 73%, respectively. After establishing these transfection efficiencies, HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were cultured (each at 1 × 10^5 cells per well) in six-well plates for 24 hours. Cultures 60%–80% confluent were transfected with a vector containing dominant negative STAT3 (Dn-STAT3; 1 μg/mL) or the empty control vector (1 μg/mL) by the use of LipofectAMINE PLUS (Life Technologies, Inc.) in serum-free medium for 3 hours at 37 °C. Cultures were incubated with complete medium for 24 hours and then stimulated with one of two doses of IL-6 (16 or 32 ng/mL) for 3 days. The Dn-STAT3-containing vector and empty vector were gifts from Dr. Koichi Nakajima (Osaka University Medical School, Osaka, Japan). The dominant negative construct carries a tyrosine-to-phenylalanine substitution at position 705 that causes a reduction of tyrosine phosphorylation of wild-type STAT3 and inhibits the action of endogenous STAT3 (23, 57).

In separate experiments, HOSE 306 and HOSE 642 cells were transfected with a vector containing wild-type STAT3 (0.25–1 μg/mL; a gift from Dr. Koichi Nakajima)—three dishes for each dose. After 72 hours, cells were harvested by scraping, washed with PBS, pelleted, and counted with a hemocytometer.

Soft Agar Assay

OVCA 429 and OVCA 432 cells were transiently transfected with the Dn-STAT3 vector or the empty vector as described above, and 24 hours later, transfected cells were cultured at 500 cells per 100-mm plate (four plates per sample) in 0.3% agar above an underlayer of 0.6% Noble agar, both containing complete medium (58). Plates were incubated for up to 4 weeks, and then the number of colonies was scored to establish the soft-agar colony-formation frequency and expressed as the number of colonies per plate cultured. Data are expressed as the mean ± 95% confidence intervals from four separate plates.

Data Analysis

Data are expressed as the mean of two to four experiments, each in triplicate samples for individual treatments or dosage regimens. Statistical analysis was carried out with a one-way analysis of variance, followed by Tukey’s post hoc test. Values are presented as the mean ± 95% confidence intervals. All statistical tests were two-sided and were considered to be statistically significant at P<.05.

RESULTS

Effect of Gonadotropins and Steroid Hormones on the Expression of IL-6 mRNA and Protein in HOSE and OVCA Cells

HOSE (HOSE 301, HOSE 306, HOSE 642, and HOSE 12–12) and OVCA (OVCA 420, OVCA 429, OVCA 432, and OVCA 433) cell lines were treated with medium alone or with 1 μM FSH, 1 μM LH, 1 μM 17β-estradiol, 1 μM estrone, 1 μM testosterone, or 1 μM 5α-dehydrotestosterone for 5 days. Relative levels of IL-6 mRNA were then measured with semiquantitative RT–PCR. Data from two cell lines from each group, HOSE 306 and HOSE 642 cells and OVCA 429 and OVCA 432
cells, are shown in Fig. 1, A. Levels of IL-6 mRNA in all HOSE and OVCA cell lines exposed to FSH, LH, 17β-estradiol, or estrone were 1.5-fold and 2.5-fold higher than in their respective untreated control cultures. Treatment of HOSE and OVCA cell lines with testosterone or 5α-dehydrotestosterone increased the expression of IL-6 mRNA in the OVCA cell lines OVCA 420, OVCA 429, and OVCA 432 but not in HOSE cell lines. OVCA 433 cells did not respond to androgens (results not shown). OVCA 433 cells do not express the androgen receptor (12) and thus cannot respond to androgen (17).

Levels of IL-6 protein were assessed in untreated and treated HOSE and OVCA cell lines by western blotting and ECL (Fig. 1, A). Gonadotropins (FSH and LH) and the two estrogens increased levels of IL-6 protein in HOSE 306 and HOSE 642 cells by 1.5-fold to 2.5-fold and in OVCA 429 and OVCA 432 cells by 3-fold to 4-fold. 5α-Dehydrotestosterone and testosterone increased the level of IL-6 protein in OVCA cell lines by two- to threefold. Overall, gonadotropins appeared to be more potent than sex steroid hormones in stimulating the expression of IL-6 in all cells.
Blocking Gonadotropin-Induced and Steroid Hormone-Induced IL-6 Expression

To ascertain whether the observed gonadotropin-stimulated or steroid-stimulated IL-6 expression is mediated through a receptor-mediated pathway, HOSE and OVCA cell lines were treated with FSH, LH, testosterone, or 17β-estradiol (each at 10 nM) for 5 days in the presence of the protein kinase A-selective inhibitor H-89 at 0.1 mM (for FSH and LH), 4-hydroxylflutamide at 0.1 mM (for testosterone), the anti-estrogen ICI 182,780 at 0.1 mM (for 17β-estradiol), or with medium alone. For all cell lines, addition of H-89 abolished the expression of gonadotropin-stimulated expression of IL-6 mRNA, and addition of 4-hydroxylflutamide or ICI 182,780 resulted in a marked decrease in testosterone-stimulated or 17β-estradiol-stimulated IL-6 mRNA expression, respectively (Fig. 1, A).

Enhanced Expression of IL-6Rα in OVCA Cell Lines

IL-6 acts through a membrane-associated receptor complex composed of the IL-6-specific subunit IL-6Rα and the signal transducer gp130, which is also used by other cytokines (30). OVCA 429 and OVCA 432 cells expressed higher levels of IL-6Rα mRNA and protein than HOSE 642 and HOSE 306 cells (Fig. 2). It is interesting that the relative abundance of gp130 mRNA in all cells was comparable. Expression patterns of IL-6Rα mRNA (Fig. 2, A) and protein (Fig. 2, B) were consistent. A twofold to threefold increase in IL-6Rα protein levels was noted in OVCA cells (P<.001).

Effect of Anti-IL-6 Antibody on IL-6-Induced Proliferation of HOSE and OVCA Cells

Fig. 2. Levels of interleukin 6 receptor (IL-6R) messenger RNA (mRNA) and protein and gp130 mRNA in normal immortalized human ovarian surface epithelial (HOSE) cells (HOSE 306 and HOSE 642) and ovarian cancer (OVCA) cells (OVCA 429 and OVCA 432). A) Total cellular RNA (1 μg) was isolated, reverse transcribed, and amplified by polymerase chain reaction (PCR). Products were separated by electrophoresis in a 2% agarose gel. Three reverse transcription (RT)-PCRs were performed from total RNA of each cell line. Relative mRNA levels are expressed as arbitrary units as described in Fig. 1. Data are the means of three experiments. Error bars are the 95% confidence intervals. Open bars = gp130 mRNA; solid bars = IL-6Rα mRNA. Statistically significant increases are indicated by asterisks (*P<.001). B) Proteins in cell lysates were separated by electrophoresis, and immunoblots were prepared and probed with an anti-IL-6Rα antibody. Data are the mean of three experiments. Error bars are the 95% confidence intervals. The relative abundance of IL-6Rα protein of the indicated cell lines is as described in Fig. 1. Statistically significant increases in IL-6Rα protein are shown by asterisks (*P<.001).

Effect of Anti-IL-6 Antibody on Hormone-Induced Proliferation of HOSE and OVCA Cells

HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were cultured with 10 nM FSH, 10 nM LH, 10 nM testosterone, 10 nM 5a-dehydrotestosterone, 10 nM estrone, or 10 nM 17β-estradiol alone or with an IL-6 antibody at 50 μg/mL for 5 days, and cell proliferation was assessed by an MTT assay. HOSE cells treated with gonadotropins (FSH and LH) or estrogens contained fourfold to fivefold more cells than untreated HOSE cells. Addition of IL-6 antibody markedly reduced HOSE cell proliferation stimulated by FSH, LH, estrone, or 17β-estradiol (Fig. 3, B). It is interesting that cell proliferation induced by testosterone or 5α-dehydrotestosterone was not inhibited by anti-IL-6 antibody in HOSE cells but was reduced by anti-IL-6 antibody in OVCA cells. Anti-IL-6 antibody alone or nonimmune immunoglobulin G (IgG) had essentially no effect on cell proliferation (results not shown).

Overexpression and Constitutive Activation of STAT3 in OVCA Cells

Binding of IL-6 to its receptor induces STAT3 phosphorylation (32); however, in many cancer cells, STAT3 is constitutively activated in the absence of IL-6 (34). Consequently, we first examined endogenous levels of phosphorylated (activated) and total STAT3 in untreated HOSE and OVCA cells by immunoblot analysis (Fig. 4, A). Blots of HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cell lysates were probed with a phosphorylation-specific STAT3 antibody or a pan-STAT3 antibody that recognizes both phosphorylated and unphosphorylated STAT3. In the absence of IL-6, OVCA cells expressed higher levels of total STAT3 protein and phosphorylated STAT3 protein than HOSE cells. OVCA cells have the main phosphorylated STAT3 band (Fig. 4, A, labeled P-STAT3α) and a faster migrating phosphorylated STAT3 isoform (labeled P-STAT3β).
The phosphorylation-specific antibody used in this study recognizes an epitope common to STAT3/H9251 and STAT3/H9252 and appears to have detected two bands with molecular masses corresponding to previously described STAT3 isoforms (97 kDa and 87 kDa, respectively). The fast migrating isoform may represent a previously reported dominant negative STAT3 isoform (59).

Levels of STAT3 Transcription Factor in OVCA and HOSE Cells

To determine the levels of STAT3-specific transcription factors in HOSE and OVCA cells, nuclear extracts were prepared from HOSE 306, HOSE 642, OVCA 432, and OVCA 429 cells. After incubation of nuclear extracts from HOSE and OVCA cells with the labeled STAT3 consensus oligonucleotide, which has been used to identify STAT3 transcription factors, complexes with approximately the same mobility were identified by radioautography (Fig. 4, B). Nuclear extracts from the two OVCA cell lines had more STAT3 binding activity than nuclear extracts from HOSE cells. In all extracts, addition of a 100-fold excess of a specific STAT3 competitor oligonucleotide completely abolished the binding of nuclear proteins to the labeled STAT3 oligonucleotide. In contrast, incubation with a nonspecific competitor did not reduce STAT3 binding. Thus, OVCA cells appear to have higher levels of STAT3 transcription factors than HOSE cells.
IL-6-Induced STAT3 Expression in HOSE and OVCA Cells

STAT3 is activated by phosphorylation. To determine the effect of IL-6 on the activity of STAT3, HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were treated with IL-6 at 32 ng/mL for 6 hours, and cell lysates were prepared for immunoblot analysis with the phospho-STAT3 antibody and the pan-STAT3 antibody. HOSE cells treated with IL-6 had a higher level of STAT3 expression than untreated HOSE cells (Fig. 4, C). In HOSE cells, phosphorylation of STAT3 was entirely dependent on the presence of IL-6, and in the absence of IL-6, STAT3 was not phosphorylated. In marked contrast, STAT3 in OVCA cells was phosphorylated in the presence or absence of IL-6, and addition of IL-6 to OVCA cells did not increase the level of total or phosphorylated STAT3.
IL-6- Stimulated Growth of HOSE and OVCA Cells and the Ectopic Expression of Dn-STAT3

To study the role of IL-6-induced STAT3 in cell proliferation, HOSE 306, HOSE 642, OVCA 429, and OVCA 432 cells were transiently transfected with 1 μg of an expression vector carrying a Dn-STAT3 construct or 1 μg of empty vector. Cultures were then treated with IL-6 (16 or 32 ng/mL) for 3 days; control cultures were incubated without IL-6 for 3 days. Proliferation in the four cell lines transfected with the empty vector was not altered (Fig. 5, A). Treatment with IL-6 increased proliferation of all cell lines in a dose-dependent manner. A greater reduction in IL-6-induced cell proliferation was noted in all cells transfected with Dn-STAT3 than in corresponding cells transfected with the empty vector (Fig. 5, A). A slight statistically nonsignificant decrease in cell proliferation was observed when HOSE cells were transfected with Dn-STAT3 alone. OVCA cells, however, transfected with Dn-STAT3 alone showed a marked decrease (P<.04) in cell proliferation (results not shown).

Overexpression of STAT3 and HOSE Cell Proliferation

To investigate whether overexpression of wild-type STAT3 affects HOSE cell proliferation, HOSE 306 and HOSE 642 cells were transfected with a vector containing wild-type STAT3, and 72 hours later, cell proliferation was assessed. A greater dose-dependent increase in cell proliferation was seen in the two wild-type STAT3-transfected HOSE cell lines than in untreated cells (STAT3 at 0.5 μg/mL in HOSE 306 cells, P<.002; STAT3 at 1.0 μg/mL in HOSE 306 or both concentrations of STAT3 in HOSE 642 cells, P<.001) or cells transfected with the empty vector alone (Fig. 5, B).

Colony-Forming Potential of OVCA Cells and the Ectopic Expression of Dn-STAT3

Transformed cells can grow in semisolid medium (e.g., soft agar). To determine whether STAT3 activation is responsible for this behavior, OVCA 429 and OVCA 432 cells were transfected with 1 μg of Dn-STAT3 DNA or 1 μg of the empty vector DNA and then plated on soft agar. OVCA cells formed fewer fewer soft-agar colonies after transfection with Dn-STAT3 than after transfection with the empty vector (OVCA 429 cells, P<.001; OVCA 432 cells, P<.006) (Fig. 5, C).

DISCUSSION

The goal of this study was to elucidate the role of hormones in the regulation of IL-6 expression and the effect of IL-6 on normal (HOSE) and malignant (OVCA) human ovarian surface epithelial cells. We capitalized on our access to several immortalized but nontumorigenic HOSE cell lines, established OVCA cell lines from late-stage serous ovarian cancer, and an expression vector carrying the dominant negative STAT3 construct (Dn-STAT3). Our results demonstrate that 1) gonadotropins and sex steroid hormones increased the expression of IL-6 in HOSE and OVCA cells, 2) an IL-6-neutralizing antibody markedly diminished gonadotropin-stimulated and sex hormone-stimulated cell proliferation of HOSE and OVCA cells, 3) IL-6 stimulated HOSE and OVCA cell proliferation in a dose-dependent, STAT3-mediated manner, 4) IL-6 induced the expression and phosphorylation (activation) of STAT3 in HOSE cells, 5) STAT3 was constitutively phosphorylated (activated) and over-expressed in OVCA cells in an IL-6-independent manner, 6) OVCA cells had a higher level of STAT3 transcriptional factors and IL-6Rα than HOSE cells, 7) blockade of STAT3 action by ectopic expression of a dominant negative STAT3 suppressed the IL-6-stimulated proliferation of HOSE and OVCA cells and the anchorage-independent growth of OVCA cells, and 8) overexpression of wild-type STAT3 increased the proliferation of HOSE cells. To our knowledge, these findings present the first evidence in support of a growth-regulatory mechanism in HOSE and OVCA cells that is stimulated by reproductive hormones and mediated by IL-6/STAT3 signaling. Defects in this signaling pathway may be involved in HOSE cell oncogenesis and confer malignant phenotypes to OVCA cells.

Epidemiologic data suggest that gonadotropins and sex steroid hormones, especially estrogens, are risk factors for epithelial ovarian cancer (3–11). However, the mechanism of hormone-induced neoplastic transformation remains unknown. We have recently demonstrated that gonadotropins, estrogens, and androgens promote cell proliferation in primary cultures of normal HOSE cells and immortalized HOSE cell lines (17). Because incessant cell proliferation is a plausible mechanism of cellular transformation (18), HOSE cell proliferation enhanced by reproductive hormones—during perimenopausal and postmenopausal periods and the use of estrogen/hormone replacement therapy—may contribute to tumor initiation. Results from this study strongly suggest that reproductive hormones may act through a convergent growth-stimulatory pathway that involves increased IL-6 expression and STAT3 activation in HOSE cells. We have demonstrated that gonadotropins and estrogens induce higher levels of IL-6 expression in HOSE cells. Increased IL-6 production after hormone treatment coincided with the observed hormone-induced proliferation, which was partially inhibited by an IL-6-neutralizing antibody. The latter finding indicates that the hormone-induced increase in cell proliferation is mediated principally by IL-6, although other growth factors may make a contribution. Enhanced IL-6 expression activates STAT3, which is associated with oncogenesis in other cancers (34). The growth-promoting effect of IL-6 on HOSE cells was clearly dependent on IL-6 and mediated by STAT3, because this effect was blocked by an IL-6-neutralizing antibody or by the ectopic expression of Dn-STAT3 in these cells. IL-6-treated HOSE cells also had higher levels of STAT3 binding factors than untreated cells. Pharmacologic concentrations of estrogens have been reported to enhance IL-6 expression in mononuclear cells in vitro (50) but to inhibit IL-6 synthesis in a human osteoblastic cell line, vascular smooth muscle cells, and bone marrow-derived stromal cells (51–53). Gonadotropins, on the other hand, have been reported to stimulate IL-6 production in Sertoli cells and in the ovari of Cynomolgus fascicularis (45,46). To our knowledge, no information was available on the hormonal regulation of IL-6 production in HOSE cells before this study. Hence, findings from this study are the first to establish that reproductive hormones stimulate HOSE cell proliferation (17) through a common signaling cascade involving IL-6 and STAT3.

Normal IL-6 signal transduction involves formation of a complex between IL-6 and its cell surface receptor, which is composed of IL-6Rα and the signal transducer gp130. IL-6Rα is specific for IL-6, whereas gp130 is also a component of cytokine receptors (30). After IL-6 signaling, Janus kinase mediates the activation of STAT3, and activated STAT3 transmits a message from the cell surface to the nucleus that turns on specific gene
Fig. 5. A) Effect of the dominant negative signal transducer and activator of transcription 3 (STAT3) and interleukin 6 (IL-6) on the proliferation of normal immortalized human ovarian surface epithelial (HOSE) cells (HOSE 306 and HOSE 642) and ovarian cancer (OVCA) cells (OVCA 429 and OVCA 432). Cells were transfected with a vector containing STAT3 (Dn-STAT3) or an empty vector, as indicated, and treated with IL-6 (16 or 32 ng/mL) for 3 days; control cells were neither transfected nor treated with IL-6. After the 3-day incubation, cells were counted with a hemocytometer, and proliferation of IL-6-treated cells was expressed as a fold increase of that of control cells. B) STAT3-induced proliferation of normal immortalized HOSE 306 and HOSE 642 cells. Cells were transfected with various concentrations of vector containing STAT3 (Dn-STAT3) or empty vector, and 72 hours later, cells were counted with a hemocytometer. Proliferation of STAT3-transfected cells is expressed as a fold increase of that of control cells. C) Dominant negative STAT3 and the colony formation ability of OVCA 429 and OVCA 432 cells. Cells were transiently transfected with a vector containing a dominant negative STAT3 (Dn-STAT3) or an empty vector. Twenty-four hours later, cells were cultured on soft agar, and 14 days later, colonies were counted. A and B) Data are the means of two experiments with triplicate samples. C) Data are the means of two experiments, with four plates for each point. Error bars are the 95% confidence intervals. Statistically significant increases (A and B) or decreases (C) in colony number are indicated with asterisks (for all cells in A, *P<.004 to .02; for B, wild-type STAT3 at 0.5 μg/mL in HOSE 306 cells, *P<.002, and for wild-type STAT3 at 1.0 μg/mL in HOSE 306 cells and both concentrations in HOSE 642 cells, **P<.001; for C, OVCA 429 cells, *P<.001, and OVCA 432 cells, **P<.006).
programs for cell proliferation and other functions. Inappropriate activation of this signal transduction pathway is found in many human cancers (34). It has been widely reported that IL-6 is elevated in serum and ascites fluid from patients with ovarian cancer, and high levels of IL-6 in body fluids are associated with poor prognosis and survival (35–37). In this study, we have identified several alterations in the IL-6/STAT3 signaling cascade in OVCA cells: 1) Higher levels of IL-6Rs were detected in OVCA cells than in HOSE cells. This observation was consistent with the slightly higher IL-6-stimulated proliferation in OVCA cells than in HOSE cells. 2) High levels of phosphorylated STAT3 were detected in OVCA cells in the absence of IL-6. 3) EMSA results demonstrated higher STAT3 binding activity in nuclear extracts of OVCA cells than in nuclear extracts of HOSE cells. 4) Inhibition of STAT3 action by the ectopic expression of Dn-STAT3 demonstrated the importance of STAT3 in maintaining OVCA cell proliferation and anchorage-independent colony formation. Thus, the IL-6/STAT3 signaling pathway is constitutively activated and is more active in OVCA cells than in HOSE cells. These findings are in agreement with those reported by Huang et al. (60), who demonstrated that the constitutive activation of STAT3 was accompanied by overexpression of Bcl-XL (i.e., the BCL2L1 gene) and cyclin D1 in several OVCA cell lines. Elevation of nuclear STAT3 binding activity has also been reported in cancers of the breast, prostate, lymphoid tissues, and the head and neck (27,34,61–63). Thus, it is now widely accepted that constitutively activated STAT3 signaling directly contributes to oncogenesis and maintenance of the malignant state (34). STAT3 regulates an array of genes, such as those for Bcl-XL, cyclin D1, p21WAF1/CIP1, and c-Myc, that are regulators of cell cycle progression and apoptosis (34). Aberrant expression of these genes, as a result of the inappropriate activation of STAT3, could promote tumor initiation and progression (62,64,65). Although constitutive action of STAT5, like STAT3, has been shown directly to result in cellular transformation, IL-6 primarily activates STAT3 and STAT1, and several studies have shown that STAT1 does not contribute to oncogenesis. We, therefore, studied only STAT3-mediated IL-6 activities in HOSE and OVCA cells.

HOSE and OVCA cells exhibited differential responses to androgen (testosterone and 5α-dehydrotestosterone)-regulated IL-6 expression. HOSE cells did not respond to either androgen by increasing the expression of IL-6. However, these two androgens do increase HOSE cell proliferation (17). Thus, androgen-induced HOSE cell proliferation appears to result from the direct action of androgen and not the indirect induction of IL-6. The inability of an IL-6-neutralizing antibody to inhibit HOSE cell proliferation supports this conclusion. It is interesting that androgens stimulated cell proliferation and the expression of IL-6 in OVCA cells. Androgen-stimulated proliferation of OVCA cells, in contrast to the proliferation of HOSE cells, could be partially blocked by an IL-6-neutralizing antibody. At present, the importance of this finding is uncertain and definitely warrants further investigation because many ovarian tumors produce androgens (66). However, studies in other cell and tissue types clearly show great variability in the mechanism of androgen-regulated IL-6 expression. For example, both testosterone and 5α-dehydrotestosterone inhibit IL-6 production in stromal cells derived from murine bone marrow (47), brain glial cells and astrocytes (48), and a human osteoblastic cell line (49). Yet, a low dose of testosterone in Sertoli cells stimulates IL-6 expression, whereas higher doses inhibit IL-6 expression (46).

In conclusion, our data demonstrate, to our knowledge for the first time, that gonadotropins and sex steroid hormones increase the expression of IL-6 in HOSE and OVCA cells. By activating STAT3, IL-6 serves as a common growth regulator for both HOSE and OVCA cells. More importantly, increased STAT3 activity may directly contribute to HOSE cell transformation and the progression of ovarian cancer. Lastly, enhanced expression of IL-6Rα, constitutive activation of STAT3, and acquired androgen regulation of IL-6 production have been identified as defects associated with ovarian malignancy. Thus, reproductive hormone-stimulated activation of the IL-6/STAT3 pathway may play a role in the pathogenesis and progression of ovarian cancer.

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NOTES

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