Cell proliferation of human ovarian cancer is regulated by the opioid growth factor-opioid growth factor receptor axis

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Donahue RN, McLaughlin PJ, Zagon IS. Cell proliferation of human ovarian cancer is regulated by the opioid growth factor-opioid growth factor receptor axis. Am J Physiol Regul Integr Comp Physiol 296: R1716–R1725, 2009. First published March 18, 2009; doi:10.1152/ajpregu.00075.2009.—Ovarian cancer is the leading cause of death from gynecological malignancies. Understanding the biology of these tumors, as well as treatment modalities, has been challenging. The opioid growth factor (OGF; [Met5]-enkephalin) and the OGF receptor (OGFr) form an endogenous growth-regulating pathway in homeostasis and neoplasia. In this investigation, we examined the relationship of the OGF-OGFr axis to ovarian cancer, and defined its presence, function, and mechanisms. Using OVCAR-3 and SKOV-3 ovarian cancer cell lines, we found that OGF and OGFr were present and functional. Exogenous OGF was observed to have a dose-dependent, serum-independent, reversible, and receptor-mediated inhibitory action on cell proliferation that was dependent on RNA and protein synthesis. The suppressive effect of OGF on cell proliferation also was observed in SW626, CAOV-3, and HEY ovarian cancer cell lines. Endogenous OGF was found to be constitutively produced and tonically active on cell replicative activities, with neutralization of this peptide accelerating cell proliferation. Silencing of OGFr using siRNA technology stimulated cell replication, documenting its integral role. The mechanism of OGF-OGFr action on DNA synthesis was related to the cyclin-dependent kinase inhibitory pathway because knockdown of p16 or p21 in OVCAR-3 cells, and p21 in SKOV-3 cells, eliminated OGF’s inhibitory effect on growth. These data are the first to report that the OGF-OGFr system is a native biological regulator of cell proliferation in human ovarian cancer. This information will be important in designing treatment strategies for this deadly disease.

ovarian cancer; cell proliferation; tissue culture; siRNA; cyclin-dependent kinase inhibitor

OVARIAN CANCER IS THE LEADING cause of death from gynecological malignancies (8), and is the 4th leading cause of cancer-related mortality among women in the United States (11). Approximately 90% of primary ovarian cancers are epithelial tumors (2). The most common presentation (75%) for epithelial ovarian cancer is in the advanced stages (stage III/IV), with cytoreductive surgery and adjuvant chemotherapy serving for treatment (7). Initial clinical response/remission for these patients is excellent, but almost 65% of patients with advanced-stage ovarian cancer relapse within 2 years of initial therapy (8). Once ovarian cancer recurs, all subsequent treatments are palliative (7). It is well recognized that defining the cellular and molecular events leading to these cancers is needed, and major improvements in ovarian cancer treatment will likely require new therapies based on exploitation of biological pathways (8).

Address for reprint requests and other correspondence: I. S. Zagon, Dept. of Neural and Behavioral Sciences, H109, The Milton S. Hershey Medical Center, 500 Univ. Drive, Rm. C3729, Hershey, PA 17033 (e-mail:isz1@psu.edu).
ies. At least three coverglasses were examined using epifluorescent microscopy.

OGFr binding assays. Receptor binding assays for OGFr were performed using log-phase cells (18, 19, 28) and custom-synthesized \([\text{[H]}-\text{Met}^-]\) enkephalin (Perkin Elmer-New England Nuclear; 52.7 Ci/mmol). Independent assays were performed at least 4 times.

Cell growth. Cells were plated and counted 24 h later (time 0) to determine seeding efficiency. OGF or other compounds were added at time 0, and media and compounds were replaced daily. All drugs were prepared in sterile water, and dilutions represent final concentrations of the compounds. An equivalent volume of sterile water was added to control wells. At designated times, cells were harvested, stained with Trypan blue, and counted with a hemacytometer. At least two aliquots per well of at least 2 wells/treatment/timepoint were sampled.

Specificity of endogenous OGF. The specificity of endogenous OGF for cell growth was determined by treating cells with a polyclonal antibody to OGF (1:200; CoI72); preimmune rabbit serum (1:200) served as a control. Serum and media were changed daily, and cells were counted after 72 h of treatment. Cell viability was determined by trypan blue exclusion at least two aliquots/well and at least 2 wells/treatment evaluated.

Specificity of OGFr knockdown with OGFr-siRNA. The OGFr-targeted siRNA (antisense, 5′-uagaaacucagugugc-3′; sense, 5′-cgcaacugagauuuc-3′) was designed and obtained as a ready-annealed, purified duplex probe from Ambion (Austin, TX). 5 × 10^4 cells/well were seeded in 6-well plates containing 1 ml of media without antibiotics. Cells were transfected with either 20 nM OGFr-siRNA or scrambled siRNA (Ambion) solutions with oligofectamine reagent (Invitrogen, Carlsbad, CA) in serum and antibiotic-free media. Cells were incubated for 4 h at 37°C before the addition of 10⁻⁶ M OGF or NTX. Cultures were incubated for an additional 20 h, and fresh complete media (2 ml) either lacking or containing OGF or NTX were added. At 72 h, cells were collected and either stained or harvested for RNA for Northern blot analysis experiments. Two independent experiments were conducted.

Total RNA was extracted using the Paris Kit (Ambion), separated on an agarose gel, and transferred to a nylon membrane (Immobilon, Bio-Rad Laboratories, Hercules, CA). Membranes were probed with 32P-DCTP-OGFr cDNA. To control for equal loading, blots were stripped and reprobed with radiolabeled GAPDH, and the optical density of each band was determined and analyzed by QuickOne (Bio-Rad Laboratories). Each value was normalized to GAPDH from control wells. At designated times, cells were harvested, stained with antibodies to BrdU (anti-BrdU-BOD, Invitrogen, Carlsbad, CA), and either counted or processed for TUNEL to assess apoptosis and necrosis (TUNEL, Trypan blue, and 0.25% trypsin-EDTA). At least two coverslips/treatment were counted, and a ratio of the number of stained cells to total cells was calculated.

To evaluate the level of OGFr protein knockdown, semiquantitative immunohistochemistry was used on a subset of cultures that were seeded and transfected with siRNAs on round coverslips. For quantification of OGFr protein levels, images were taken at the same exposure time with special care not to photobleach samples. The mean intensity of staining was determined for at least 100 cells/group, and at least 2 coverslips/group.

Mechanisms of OGF-modulated growth inhibition: DNA synthesis, apoptosis and necrosis. The effect of OGF on DNA synthesis (BrdU incorporation), apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL), and necrosis (TUNEL, Trypan blue) of human ovarian cancer cells were evaluated. Cultures were seeded onto 22-mm diameter coverslips placed in 6-well plates (5 × 10^4 cells/coverglass), and treated with compounds for 72 h, with media and drugs replaced daily. Three hours prior to fixing cells, 30 μM BrdU (Sigma Chemicals, Indianapolis, IN) was added to some cultures. Cells were fixed in 10% neutral buffered formalin for 10 min, and either stained with antibodies to BrdU (anti-BrdU-BOD, Invitrogen) to assess DNA synthesis, or processed for TUNEL to assess apoptosis and necrosis.

OGF and the pathway of cell cycle inhibition. OVCAr-3 and SKOV-3 cells (6 × 10^5) were synchronized with 50 nM nocodazole (Sigma-Aldrich, St. Louis, MO) for 24 h, followed by three washes with complete media to release cells from growth arrest. To monitor p16 or p21 expression, cells were treated with sterile water or 10⁻⁴ M OGF for 1 to 24 h. Cells were harvested with 0.25% trypsin-EDTA (Mediatech, Herndon, VA) and solubilized in 200 μl RIPA buffer (1× PBS, 10 μM IGEPAL, 1 mg/ml SDS, 5 mg/ml deoxycholic acid), containing protease and phosphatase inhibitors (2 μg/ml apro- tinin, 3 mg/ml phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 1 μM okadaic acid). Total protein concentrations were measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Equal amounts of protein (40 μg) were subjected to 20% SDS-PAGE followed by transfer of proteins onto nitrocellulose using standard protocols. The following antibodies were purchased from commercial sources: p16 (BD Pharmingen, San Diego, CA), p21 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Clone AC-15, Sigma-Aldrich). Membranes were probed with p16 or p21 antibodies (1:200), followed by secondary anti-mouse horseradish peroxidase conjugated antibodies (GE Healthcare-Amersham Biosciences, Piscataway, NJ; 1:5,000), and developed using a chemiluminescent Western blotting detection system. To determine equal loading of total protein, blots were stripped with stripping buffer (62.5 mM Tris·HCl and 100 mM β-mercaptoethanol/2% SDS, pH 6.7) at 50°C, and reprobed with monoclonal antibody to β-actin (1:5,000).

The optical density of each band was determined by densitometry (QuickOne), and each value was normalized to β-actin from the same blot. The fold increase was calculated by dividing the normalized value from the OGF-treated samples by the normalized value of control samples at time 0. Means and SE were determined from at least two independent experiments.

siRNA knockdown of p16 and p21. Log-phase cells were transfected with 20-nM concentrations of p16 and/or p21 siRNA’s (Santa Cruz Biotechnology), or scrambled siRNA’s (Ambion) with Oligo-fectamine reagent (Invitrogen) in serum and antibiotic-free media for 4 h at 37°C before the addition of OGF (10⁻⁷ M). Cultures were incubated an additional 20 h, and then 2 ml fresh complete media lacking or containing OGF were added. OGF and media were changed daily. At 72 h after the start of transfection, cells were collected for Western blot analysis to determine the level of p16 and p21 knockdown.

OGF action and protein and RNA synthesis. To examine whether OGF’s inhibitory and NTX’s stimulatory effects are dependent on protein and RNA synthesis, log-phase cells were treated with 5 μg/ml concentrations of cyclohexamide or puromycin for protein synthesis, or actinomycin D for RNA synthesis, 10⁻⁶ M concentrations of OGF or NTX, or an equivalent volume of sterile water, and 30 μM BrdU for 3 h. Cells were rinsed, fixed in 10% formalin for 10 min, and stained with antibodies to BrdU. At least 1,000 cells/treatment on at least 3 coverslips/treatment were counted, and a ratio of the number of positive cells divided by total cells was calculated.

Chemicals. The following compounds were obtained from the indicated sources: [Met⁵]-enkephalin, [Leu⁵]-enkephalin, [α-Pen⁵]-enkephalin (DPDPE), [α-Ala³-MePhe⁶-Gly⁷]-enkephalin (DAMGO), β-endorphin, naltrexone (NTX), naloxone (NAL), dynorphin A1–8, morphine sulfate, endomorphin 1, endomorphin 2 (Sigma), and U69,593 (Upjohn Diagnostics, Kalamazoo, MI).

Statistical analysis. All data were analyzed (GraphPad Prism software) using one-way ANOVA, with subsequent comparisons made using Newman-Keuls tests.

RESULTS

OGF and OGFr are present in human ovarian cancer cells. Epifluorescent microscopy revealed immunoreactive OGF and OGFr in both the cytoplasm and nucleus of OVCAr-3 and SKOV-3 cells. OGF was particularly prominent in the perinuclear cytoplasm of OVCAr-3 cells but appeared to contain less nuclear staining compared with SKOV-3 cells (Fig. 1A). No
staining was recorded in specimens processed with secondary antibody only.

Receptor binding analysis of nuclear protein from log-phase OVCAR-3 and SKOV-3 cells revealed site-specific and a one-site model of saturable binding (Fig. 1B). The binding capacities (Bmax) for OVCAR-3 and SKOV-3 were 4.5 ± 0.5 and 3.9 ± 0.2 fmol/mg protein, respectively, whereas the binding affinities (Kd) for OVCAR-3 and SKOV-3 were 4.2 ± 1.0 and 6.2 ± 1.9 nM, respectively.

OGF depresses growth of ovarian cancer cells. The effects of OGF in concentrations ranging from 10^{-4} M to 10^{-10} M at 72 h revealed a dose-dependent inhibitory effect on cell proliferation (Fig. 2A). Dosages of 10^{-4} M to 10^{-9} M, but not 10^{-10} M, reduced cell number in OVCAR-3 cultures by 16–51%, and SKOV-3 cultures by 10–36%, from vehicle-treated groups. Over a 120-h period, cell number was reduced significantly in OVCAR-3 cultures treated with 10^{-6} M OGF by 20% to 27%, and in SKOV-3 cultures by 13% to 23%, compared with control cultures (Fig. 2B).

To determine whether OGF activity was mediated by an opioid receptor, cells were grown in the presence of OGF and the short-acting nonselective opioid receptor antagonist naloxone (Nal) at a concentration of Nal that did not influence cell proliferation (Fig. 2C). Cell number was reduced by 30% in both OVCAR-3 and SKOV-3 cultures treated with 10^{-6} M OGF compared with vehicle-treated cultures; cultures receiving both OGF and Nal, or Nal alone, did not deviate from vehicle-treated control levels.

To examine the reversibility of OGF inhibition on cell number, cultures of OVCAR-3 and SKOV-3 cells were exposed for 72 h to 10^{-6} M OGF, and cell number was decreased by 19% and 23%, respectively, from control levels. Media were removed at 72 h, and fresh media were added with no OGF (OGF-Co); some cultures continued to receive a daily change of media and OGF (OGF). At 24 h and 48 h after OGF-containing media was replaced with fresh media, cell number in the OGF-Co group in OVCAR-3 and SKOV-3 cultures was increased 9% and 13% from cultures continuing to receive OGF (i.e., OGF) (Fig. 2D).

To examine whether there were confounding variables introduced by growing cells in 10% serum, OVCAR-3 cells were adapted over a 4-wk period to low-serum media (1.5%); cells were not viable below this serum concentration. OVCAR-3 cells in 1.5% serum and treated with 10^{-6} M OGF were decreased in number by 26% from vehicle-exposed cultures (data not shown).

Fig. 1. The presence and distribution of opioid growth factor (OGF) and OGF receptor (OGFr) in human ovarian cancer cells. A: photomicrographs of log-phase OVCAR-3 and SKOV-3 cells visualized with differential interference or immunohistochemistry of samples stained with polyclonal, ammonium-sulfate purified antibodies (1:100) to [Met 5]-enkephalin (OGF) or OGFr. Rhodamine-conjugated IgG (1:1,000) served as the secondary antibody. Immunoreactivity was associated with the cytoplasm, and a speckling of stain was noted in cell nuclei. Immunostaining was not detected in cell preparations incubated with secondary antibodies only (inset). Scale bar = 10 μm. B: representative saturation isotherm of specific binding of [3H]-[Met 5]-enkephalin to nuclear homogenates of OVCAR-3 and SKOV-3 cells. Means ± SE binding affinity (Kd) and maximal binding capacity (Bmax) from at least four independent assays performed in duplicate. Representative Scatchard plot (inset) of specific binding of radiolabeled [Met 5]-enkephalin to OVCAR-3 and SKOV-3 proteins revealed a one-site model of binding for each cell line.
The endogenous-opioid specific for growth inhibition of ovarian cancer cells is OGF. To determine whether endogenous or exogenous opioids other than OGF modulate the growth of ovarian cancer cells, OVCAR-3 and SKOV-3 cultures were treated daily for 72 h with 10^{-6} M concentrations of natural and synthetic opioid-related compounds, many specific for μ, δ, and κ opioid receptors (Fig. 3A). Under the same conditions and concentrations, whereby OGF markedly decreased cell number, all other opioid-related peptides had no effect on the proliferation of either cell line.

Persistent opioid receptor blockade between OGF and OGFr with the general opioid receptor antagonist NTX (10^{-6} M) was also evaluated for its effect on cell growth of human ovarian cancer cells. OVCAR-3 and SKOV-3 cell numbers were increased 19% and 27%, respectively, from control values (Fig. 3A).

The specificity of endogenous OGF’s inhibitory action was investigated by neutralizing native OGF with a polyclonal antibody. OVCAR-3 and SKOV-3 cultures exposed to the OGF antibody had 46% and 26%, respectively, more cells than control cultures; cultures treated with sterile water (Co) and those receiving preimmune serum (IgG) had a similar number of cells (Fig. 3B).

Silencing of OGFr in human ovarian cancer cells blocks the inhibitory action of endogenous and exogenous OGF, and the stimulatory action of NTX. The requirement of the OGF receptor for OGF’s inhibitory action on cell proliferation was eval-

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**Fig. 2.** OGF inhibits growth of human ovarian cancer cells in a dose-dependent, temporal, receptor-mediated, and reversible manner. A: growth of OVCAR-3 cells and SKOV-3 cells subjected to various concentrations of opioid growth factor (OGF) for 72 h. OGF or an equivalent volume of sterile water (Co) was added 24 h after seeding 100,000 cells, and media and OGF were replaced daily. B: growth of OVCAR-3 and SKOV-3 cells treated with OGF (10^{-6} M) or an equivalent volume of sterile water over a 120-h period. OGF or water (Co) was added 24 h (0 h) after cells were seeded at 100,000 cells/well; media and OGF were changed daily. C: opioid receptor mediation of the growth inhibitory effects of OGF in OVCAR-3 and SKOV-3 cells. Cell cultures were subjected to OGF (10^{-6} M), the opioid antagonist naloxone (Nal; 10^{-6} M), OGF, and Nal, or sterile water (Co) for 72 h. Cells were seeded at 100,000 cells, and media and compounds were replaced daily. D: reversibility of the growth inhibitory effects on OVCAR-3 and SKOV-3 cells treated with 10^{-6} M OGF or sterile water (Co). Cells were seeded at 100,000/well into 6-well plates and treated with OGF or sterile water for 72 h; cells were counted at 0, 24, 48, 72, 96, and 120 h. At 72 h, one-half of the culture plates continued to receive OGF for an additional 48 h, and one-half of the plates were treated with sterile water for 48 h (OGF-Co). Control (Co) cultures received sterile water throughout the 120 h. Compounds and media were replaced daily. For all experiments, data are expressed as means ± SE for at least 2 aliquots/well from at least 2 wells/group. Significantly different from respective controls at *P < 0.05, **P < 0.01, ***P < 0.001. Significant difference between the OGF-Co and OGF groups at **P < 0.001.
Fig. 3. OGF is the specific opioid peptide involved in the growth inhibition of human ovarian cancer cells. A: effects of various endogenous and exogenous opioids on OVCAR-3 and SKOV-3 cell number. Cells were seeded at 50,000/well into 24-well plates and treated daily with 10^{-6} M concentrations of a variety of opioids for 72 h. B: OVCAR-3 and SKOV-3 cells were treated with a polyclonal antibody specific for OGF (Co172), preimmune serum (IgG), or exogenous OGF (10^{-6} M); antibodies and peptide were replaced daily. Cell number was measured at 72 h. Data for both experiments represent means ± SE for at least two aliquots/well from at least two wells/group. Significantly different from respective controls at ***p < 0.001.

OGF's inhibitory effect, but not NTX's stimulatory action, is dependent on protein and RNA synthesis. OGF's inhibitory effects on cell proliferation were absent in OVCAR-3 and SKOV-3 cultures treated with cyclohexamide, puromycin, or actinomycin D (Fig. 5, A–C). In contrast, NTX's accelerating effects on cell proliferation in these ovarian cancer cells persisted in the presence of cyclohexamide (Fig. 5A), puromycin (Fig. 5B), or actinomycin D (Fig. 5C).

p16/p21 is required for OGF-induced growth inhibition. To evaluate whether the mechanism of OGF activity is related to the induction of CKIs p16 and/or p21 expression, OVCAR-3 and SKOV-3 cells were synchronized by nocodazole (50 nM) for 24 h and subsequently treated with 10^{-6} M OGF or sterile water (vehicle). p16 protein expression in OVCAR-3 cells was upregulated compared with vehicle-treated cultures by 2.6-fold following 1 h of OGF-treatment, and 4.1-fold after 9 h of OGF exposure (Fig. 6, A and B), whereas p21 protein expression was up-regulated 2.8-fold from vehicle-treated cultures by 5 h of OGF exposure. p21 protein expression in SKOV-3 cells was up-regulated at 4, 5, 6, and 9 h by 1.4- to 2.3-fold (Fig. 6, A and B); p16 protein was not detected.

To test the role of p16 and/or p21 in OGF-induced inhibitory action in ovarian cancer cell growth, cells were treated with scrambled siRNA, p16 siRNA, p21 siRNA, or both p16 and p21 siRNA. Cells transfected with p16 or p21 siRNA had significantly reduced levels of p16 or p21 protein compared with untransfected cells after 72 h (Fig. 6C). Growth analysis of ovarian cancer cells transfected with p16 and/or p21 siRNA’s revealed that either p16 or p21 induction is required for OGF inhibitory action in OVCAR-3 cells (Fig. 6D). For SKOV-3 cells, p21, but not p16, was required to induce OGF inhibitory action (Fig. 6D). OVCAR-3 cells that were transfected with scrambled siRNA, p16 siRNA, or p21 siRNA and treated with OGF (10^{-6} M) for 72 h had reductions in growth from 33–55% compared with cells subjected to sterile water. SKOV-3 cells transfected with scrambled siRNA or p16 siRNA and exposed to OGF (10^{-6}
OGFr is required for OGF’s inhibitory action on growth. A: Northern blot analysis and semiquantitative densitometry demonstrating the specificity and level of OGFr knockdown in OVCAR-3 and SKOV-3 cells. Log phase cells were transfected for 24 h with either scrambled siRNA or OGFr siRNA. Forty-eight hours after transfection, cells were harvested and RNA isolated. Data (percent of OGFr/GAPDH ratio) represent means ± SE for 2 blots from independent experiments. Significantly different from nontransfected cultures at *P < 0.05. 

B: photomicrographs of log phase OVCAR-3 and SKOV-3 cells stained with a polyclonal antibody to OGFr (Bo344), demonstrating the extent of OGFr protein knockdown. Cells were transfected for 24 h with OGFr siRNA or scrambled siRNA and incubated in media for an additional 48 h. Photomicrographs of cells stained with OGFr were taken at the same exposure time. Semiquantitative measurement of the OGFr immunohistochemistry demonstrated the level of protein knockdown in OVCAR-3 and SKOV-3 cells. Decreased OGFr staining intensity (mean gray value) is indicative of decreased OGFr protein expression. Data represent means ± SE. Significantly different from nontransfected cells at ***P < 0.001. 

C: growth of OVCAR-3 and SKOV-3 cultures transfected with OGFr siRNA or scrambled siRNA for 24 h and treated with either OGF (10⁻⁶ M), NTX (10⁻⁶ M), or an equivalent volume of sterile water for 72 h; compounds and media were changed daily. Values are presented as means ± SE cell counts for at least 2 aliquots/well and least two wells/treatment. Significantly different at *P < 0.05 or ***P < 0.001 from cultures that were not transfected.
The ubiquity of OGF’s inhibitory action in human ovarian cancer cells was examined in three additional cell lines, SW626, CAOV-3, and HEY. After 72 h of treatment with OGF, SW626, CAOV-3, and HEY cells were reduced in number by 25%, 18%, and 24%, respectively, from their controls. Exposure to NTX increased cell number by 28%, 19%, and 29% in SW626, CAOV3, and HEY cell lines, respectively, relative to cells treated with sterile water (data not shown).

DISCUSSION

A previous study in our laboratory detected OGF by radioimmunoassay in surgical specimens of human ovarian neoplasms (22). This observation prompted two questions: 1) Does the OGF-OGFr axis exist in human ovarian cancer? and 2) Can you modulate cell proliferation in these tumor cells through the OGF-OGFr pathway? Using a tissue culture model, both OGFr and OGF were detected by immunohistochemistry, and receptor binding studies revealed that the OGF receptor was capable of binding OGF. Cell proliferation assays with OVCAR-3 and SKOV-3 cell lines ascertained that OGF depressed cell number in a dose-dependent fashion, including a dosage of drug that was of physiological relevance to the binding affinity (i.e., $10^{-9}$ M). OGF inhibitory activity was rapid, persistent, and did not exhibit tolerance, being detected as early as 24 h after initiating peptide administration and extending for 5 days. The effects of OGF on ovarian cancer cell replication were receptor mediated (blocked by naloxone in the absence of an effect of naloxone alone), reversible (indicating a cytostatic but not...
toxic action), and not dependent on serum concentration. Cells subjected to a wide variety of synthetic and natural opioids, including those specific for μ (DAMGO, endomorphin I and 2), δ (DPDPE) and κ (U69593) opioid receptors, showed that none of these compounds had any effect on growth at a concentration (10⁻⁶ M) of OGF that markedly depressed cell proliferation. Finally, the present study revealed that the OGF-OGFr axis functions in at least five different human ovarian cancer cell lines, indicating the ubiquity of the system with respect to this disease. Thus, these results reveal for the first time, the presence and significance of a native biological pathway regulating cell proliferation in human ovarian cancer.

Even though OGF was the only opioid peptide to inhibit ovarian cancer cell proliferation, and OGF was detected in these cells by immunohistochemistry, the question can be raised as to whether endogenous OGF is a functional native peptide related to growth. Two pieces of evidence support this to be the case. First, cells exposed to the general opioid antagonist NTX accelerated in growth, indicating that the opioid related to cell proliferation is constitutively expressed and tonically active. Second, and more specific to OGF, neutralization of OGF by an antibody to this peptide stimulated cell replication. Thus, depletion of endogenous OGF in these cultures removed the inhibitory influence on these cells that establishes an equilibrium in cell proliferation, thereby upsetting the delicate balance in regulating cell number by peptide activity.

An extensive literature shows that OGF interacts with OGFr to regulate cell proliferation (1, 4–6, 14–16, 18–21, 27, 28, 30), implying that effects on the control of cell number by

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**Fig. 6.** The OGF-OGFr axis in human ovarian cancer cells inhibits DNA synthesis and targets the p16 and p21 pathways. A: OGF-induced p16 and p21 expression. OVCAR-3 and SKOV-3 cells were synchronized by nocodazole (50 nM) for 24 h and subsequently treated with 10⁻⁶ M OGF or an equivalent volume of sterile water for 1 to 24 h. Total proteins were resolved by SDS-PAGE and blotted with p16 or p21 specific antibodies. B: densitometric analysis of the Western blots was done, and p16 and p21 expression for OGF-treated cells is expressed relative to controls at time 0. The p16 and p21 levels were significantly elevated from water-treated cells at *P < 0.05, **P < 0.01, or ***P < 0.001. Data are presented as means ± SE from two independent experiments. C: OVCAR-3 and SKOV-3 cells were transfected for 24 h with p16 and/or p21 siRNA, and total proteins were isolated 48 h after transfection, separated by SDS-PAGE, and probed with antibodies specific to p16 or p21 to demonstrate level of protein knockdown. D: p16 or p21 pathways are required for OGF’s inhibitory action on cell proliferation. OVCAR-3 and SKOV-3 cells were transfected with p16 siRNA, p21 siRNA, p16 and p21 siRNA, or scrambled siRNA for 24 h and subsequently treated with 10⁻⁶ M OGF or an equivalent volume of sterile water for 72 h. Data are presented as means ± SE cell counts for at least 2 aliquots/well and at least 2 wells/treatment. Significantly different at ***P < 0.001 from cultures that were not transfected and treated with sterile water.
endogenous OGF, as well as exogenously administered peptide, are mediated by this receptor. OGFr was detected in ovarian cancer cells by immunohistochemistry and receptor binding techniques; however, OGF is [Met⁵]-enkephalin, and this opioid peptide is known to bind to classical opioid receptors such as μ, δ, and κ as well (13). To examine the specificity of OGF for OGFr with respect to regulation of cell proliferation, the effect of silencing OGFr using siRNA technology was undertaken. Cells treated with OGFr siRNA were observed to have an increase in cell number, suggesting that attenuating OGFr compromised the action of endogenous OGF. Moreover, exogenously administered OGF, which depresses cell number in log-phase cultures did not have any effect when the cells were transfected with OGFr siRNA. In fact, there was a greater number of cells in cultures treated with OGFr siRNA and exposed to OGF than in untransfected cultures or those transfected with scrambled siRNA. An interesting observation in these studies is that although the use of NTX, a general opioid receptor blocking agent, increased cell number, knockdown of OGFr by OGFr siRNA was significantly more effective. These results are consistent with earlier findings using immunoelectron microscopy showing that there is still some OGF-OGFr activity even with treatment of NTX, indicating a “leakiness” with NTX blockade (26). That NTX is not the most specific blocker of OGF-OGFr interaction is understandable because the OGF receptor has nucleotide and protein sequences that are not in keeping with classical opioid receptors (30). Thus, NTX appears to have recognition of OGFr but may not provide a complete blockade of OGF-OGFr interfacing. All of these data support OGFr as the receptor mediating OGF action. Taken together with the knowledge that OGF is the peptide involved with modulating cell number of human ovarian cancer cells, it is clear that proliferation of these carcinoma cells is dependent on the OGF-OGFr axis.

A decrease in cell number as seen by treatment with OGF could be due to a decrease in cell survival because of either programmed cell death or necrosis, and/or a reduction in DNA synthesis and subsequent cell replication. Our data show that neither apoptosis nor necrosis are involved with OGF activity in ovarian cancer cells, a result consistent with a previous publication documenting a similar finding in a variety of cancer cells growing in tissue culture (23). However, DNA synthesis in cultures treated with OGF exhibited a marked diminishment from control levels. Consistent with previous studies, these data indicate that the mechanism of OGF involves regulation of cell proliferation (1, 4–6, 18–20, 27, 28).

The effects of OGF and NTX on cell proliferation beg the question of whether these modulatory effects require RNA and/or protein synthesis. We found that the inhibitory action of OGF on DNA synthesis could not be observed in the presence of inhibitors of either RNA or protein synthesis. However, the stimulatory effect of NTX on DNA synthesis persisted when cultures were treated with NTX in the presence of inhibitors of protein or RNA synthesis. These novel data indicate that OGF depends on new protein and RNA synthesis to exert its inhibitory effects on DNA synthesis, while NTX’s stimulatory effects on DNA synthesis are independent of protein and RNA synthesis.

With respect to the mechanism of OGF activity on inhibiting DNA synthesis, previous studies in pancreatic and squamous cell carcinoma, as well as normal cells, have shown regulation by p16 and/or p21 (4–6), suggesting that CKI pathways are a target of OGF. Given these results, we raised the question of whether OGF targets the p16/p21 pathways in ovarian cancer using a cell line that contains both p16 and p21 (OVCAR-3), as well as a cell line that lacks p16 but contains p21 (SKOV-3). We now demonstrate that OGF treatment in human ovarian cancer cells upregulates both p16 and p21 protein in a cell line that contains both of these proteins, and only p21 protein in a cell line that lacks p16. To evaluate whether OGF’s inhibitory action in an ovarian cancer cell line expressing both p16 and p21 requires both p16 and p21, or either p16 or p21, OVCAR-3 cells were transfected with p16 and/or p21 siRNA’s and treated with OGF. In contrast to the finding in normal cells (6), where both p16 and p21 are required for OGF action, we found that either p16 or p21 is sufficient to exert OGF’s inhibitory effects in ovarian cancer. Similar to the finding in pancreatic cancer cells (4), ovarian cancer cells lacking p16, relied on the p21 CKI pathway to exert OGF’s action. Thus, without knowledge of which CKI is present in ovarian cancer cells, a combined inhibition of p16 and p21 might be considered in designing therapeutic strategies.

Perspectives and Significance

These results reveal for the first time the presence and functional significance of a native biological pathway regulating cell proliferation in human ovarian cancer. A critical question that needs to be addressed in future studies is whether OGF has efficacy in modulating the incidence and progression of human ovarian cancer in vivo. Therefore, preclinical experiments are needed to establish whether this agent can alter the course of these lethal neoplasias.

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REFERENCES


