PATHWAYS TARGETED BY THE OGF-OGFr AXIS ARE DETERMINANTS IN THE PROGRESSION OF HUMAN OVARIAN CANCER

A Dissertation in

Cell and Molecular Biology

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2011
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Abstract

Ovarian cancer is the leading cause of death from gynecological malignancies, and is the 5th leading cause of cancer related mortality among women. An estimated 225,000 new cases are diagnosed each year worldwide, resulting in 140,200 deaths annually. Approximately 90% of primary ovarian cancers are epithelial in origin, and the most common presentation (75%) is in the advanced stages (stage III/IV). Cytoreductive surgery and adjuvant chemotherapy serve as the major treatment modalities. Initial clinical response is excellent; however, nearly 65% of advanced-staged patients relapse within 2 years of initial therapy. Once ovarian cancer recurs, all subsequent treatments are palliative. The cellular and molecular events involved in ovarian cancer pathogenesis need to be defined, and major improvements in treatment will require new therapies based on exploitation of biological pathways.

Dysregulation of cell proliferation is an integral part of the ovarian cancer phenotype. One native biological regulatory system of cell replication in normal cells and a variety of cancers is the opioid growth factor (OGF) and its receptor, OGFr. Chemically termed [Met\(^5\)]-enkephalin, OGF is a constitutively active opioid peptide that is autocrine produced and secreted, and interacts with OGFr to delay the G\(_1\)/S interface of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways, without affecting cell survival. The studies depicted in this thesis were aimed at establishing the role of the OGF-OGFr axis in the modulation of cell proliferation and determining the repercussions of modulating this axis on human ovarian cancer both in vitro and in vivo.

The first study evaluated the presence, mechanism, and role of the OGF-OGFr axis on the modulation of the growth of OVCAR-3 and SKOV-3 human ovarian cancer cell lines. 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 repressive effect of OGF on cell proliferation also was also observed in 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 by siRNA technology
stimulated cell replication, and abolished the inhibitory actions of exogenous OGF, documenting its integral role in mediating the effects of endogenous and exogenous OGF. The mechanism of OGF-OGFr action on DNA synthesis was related to the CKI pathways because knockdown of p16 and 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 cells using an in vitro model system.

The next study determined whether OGF inhibits tumor growth in mice with established subcutaneous xenografts of ovarian cancer, and determined whether the combination of OGF biotherapy with taxol or cisplatin chemotherapy (the standard of care) provides an additive inhibitory effect on ovarian cancer proliferation and tumor growth. Cell proliferation assays on SKOV-3 cells treated with OGF and/or taxol, or OGF and/or cisplatin, demonstrated that cell number and DNA synthesis were inhibited to a greater extent by combination treatments compared to individual treatments. The action of OGF, but not taxol or cisplatin, was mediated by a naloxone sensitive receptor, and was reversible and not toxic. Tumor volumes in mice with established subcutaneous xenografts were reduced up to 50% by OGF, up to 50% by taxol, and up to 58% by cisplatin treatment, compared to saline administered controls. Tumor volumes from the OGF plus taxol group were decreased 28% from mice treated with OGF alone, and 29% from mice treated taxol alone; tumor volumes in mice treated with OGF plus cisplatin were reduced 50% from mice treated with OGF alone, and 44% from mice treated with cisplatin alone. Importantly, OGF treatment in mice receiving cisplatin provided a significant degree of protection against the toxic effects of cisplatin (weight loss) seen in mice receiving cisplatin treatment alone. Evaluation of tumor tissue after 37 days of treatment revealed that taxol and cisplatin, but not OGF, induced apoptosis, while all treatments, markedly inhibited DNA synthesis and tumor angiogenesis; an additive inhibitory effect on DNA synthesis and angiogenesis was seen in mice receiving OGF with chemotherapies compared to mice receiving chemotherapy alone. Both OGF and OGFr were detected in tumor tissue, and OGFr binding and expression was reduced up to 51% and 81%, respectively, by OGF treatment, revealing a feedback mechanism. This preclinical evidence, demonstrates
that OGF biotherapy markedly inhibits ovarian tumor growth in a non-toxic manner \textit{in vivo}, and can be combined with taxol or cisplatin chemotherapy to provide an enhanced therapeutic benefit.

We next investigated the repercussions of pharmacologically manipulating the OGF-OGFr axis in human ovarian cancer cells \textit{in vitro} using the opioid receptor antagonist naltrexone (NTX), which is a nonselective opioid receptor antagonist that blocks the interaction of OGF and OGFr. It is well understood that the response to opioid antagonist administration is a compensatory upregulation in the production of endogenous opioids/opioid receptors. Pharmacokinetic and nociceptive studies have revealed that administration of a low dosage of NTX (LDN) \textit{in vivo}, which blocks endogenous opioids from opioid receptors for a short period of time (4–6 h) each day, provides a window of 18–20 h for the upregulated opioids and opioid receptors to interact. \textit{In vivo} daily administration of a low dosage of NTX (LDN, 0.1 mg/kg) has been shown to inhibit progression of colorectal cancer and neuroblastoma in xenograft models of cancer and is suggested to target cell proliferation. A tissue culture model of LDN, using short term NTX treatment, was established to investigate the mechanism of LDN in human ovarian cancer and to determine whether its action is independent of immune function and systemic factors. A single application of short term NTX ($10^{-5}$ M, 6h) to SKOV-3 cultures inhibited cell number by 21-28% between 48 and 96 h. To determine which opioid peptide(s) mediate(s) the inhibitory actions of LDN on growth, cultures were exposed to various opioid peptides; under conditions and concentrations where OGF ($10^{-6}$ M) inhibited cell number, none of the opioid peptides specific for the classical $\mu$, $\delta$, or $\kappa$ opioid receptors altered growth. To establish whether the inhibitory actions of LDN are invoked by endogenous OGF, as well as determine the involvement of a particular opioid receptor, an OGF neutralizing antibody, and siRNA’s against the $\mu$, $\delta$, $\kappa$, and OGFr receptors were administered in combination with short term NTX; neutralizing OGF and silencing of OGFr, but not $\mu$, $\delta$, or $\kappa$ opioid receptors, blocked the inhibitory action of LDN. An evaluation of the effects of short term NTX on the expression of OGF (RT-PCR, Immunohistochemistry, radioimmunoassay) and OGFr (Northern blot, Western blot, Immunohistochemistry, receptor binding) revealed a translational, but not transcriptional, upregulation of both peptide and receptor. To
ascertain whether LDN’s inhibitory action is related to decreased survival and/or proliferation, TUNEL, trypan blue staining, and BrdU incorporation were evaluated. Beginning 12 h and persisting 66 h after a single application of short term NTX, DNA synthesis was decreased up to 48%, with no alterations in cell survival noted. Silencing of p16 and/or p21 blocked the inhibitory action of short term NTX indicating a mechanism directed to the CKI pathways. This study demonstrated that short term opioid receptor antagonism, the equivalent of LDN, inhibited ovarian cancer cell proliferation independent of immune function and systemic factors, through upregulation of the OGF-OGFr axis. Upregulation of the OGF-OGFr axis by short term opioid receptor blockade with LDN may provide a novel target for biotherapy of ovarian cancer.

The next study evaluated whether LDN inhibits tumor growth in mice with established subcutaneous xenografts of ovarian cancer, and determined whether the combination of LDN biotherapy with taxol or cisplatin chemotherapy (the standard of care) provides an additive inhibitory effect on ovarian cancer proliferation and tumor growth. Cell proliferation assays on SKOV-3 cells treated with LDN and/or taxol, or LDN and/or cisplatin, demonstrated that cell number and DNA synthesis rates were inhibited to a greater extent by combination treatments compared to individual treatments. The action of LDN, but not taxol or cisplatin, was reversible and not toxic. Tumor volumes in mice with established subcutaneous xenografts were reduced up to 48% by LDN, up to 54% by taxol, and up to 54% by cisplatin treatment, compared to saline administered controls. Tumor volumes from the LDN plus taxol group, were comparable to those in mice treated with LDN or taxol alone; however, tumor volumes in mice treated with LDN plus cisplatin were reduced 35% from mice treated with LDN alone, and 37% from mice treated with cisplatin alone. Importantly, LDN treatment in mice receiving cisplatin provided a significant degree of protection against the toxic effects of cisplatin (weight loss) seen in mice receiving cisplatin treatment alone. Evaluation of tumor tissue after 35 days of treatment revealed that taxol and cisplatin, but not LDN, induced apoptosis, while all treatments, markedly inhibited DNA synthesis and angiogenesis; an additive inhibitory effect on DNA synthesis and angiogenesis was seen in mice receiving LDN with cisplatin, but not LDN with taxol, compared to mice receiving chemotherapy alone. Both OGF and OGFr were detected in tumor tissue.
Revealing the mechanism and compensatory upregulation of the OGF-OGFr axis, OGF expression was increased 37% by LDN treatment, and OGFr binding and expression was increased up to 133% and 46%, respectively, by LDN treatment, compared to saline administered controls. This preclinical evidence, demonstrates that LDN biotherapy markedly inhibits ovarian tumor growth in a non-toxic manner in vivo, and can be combined with cisplatin, but not taxol chemotherapy to provide an enhanced therapeutic benefit.

We next investigated the effects of OGF or LDN on ovarian cancer tumor growth using what is considered to be a more clinically relevant model of human ovarian cancer, where cancer cells are injected and grown in the intraperitoneal cavity as opposed to subcutaneously. Immediately after tumor cell inoculation, mice were treated with OGF, LDN, or an equivalent volume of saline. At the end of 40 days of treatment, the number of tumor nodules was decreased 41% and 38% by OGF and LDN, respectively, and tumor mass was reduced 68% and 46%, by OGF and LDN, respectively, compared to saline administered controls. This preclinical evidence, using the more clinically relevant ovarian cancer model, suggests that OGF or LDN biotherapy markedly inhibits ovarian tumor growth by inhibiting tumor cell proliferation and angiogenesis, without inducing apoptosis.

Molecular regulation of tumor cell progression was studied in two independent experiments whereby constructs to overexpress OGFr or underexpress OGFr were established and characterized both in culture and subsequently in nude mice. Using SKOV-3 cells, 5 clonal lines overexpressing OGFr were examined and demonstrated increases in OGFr protein expression, as measured by semiquantitative immunohistochemistry and Western immunoblotting. OGFr binding assays of clonal lines revealed 51-154% increases in binding capacity compared to wild-type (WT) and empty vector (EV) controls; binding affinity was comparable in all groups. Under standard growth conditions, cell number in clonal lines overexpressing OGFr was decreased by 31-85%, and doubling times were extended 41-177% compared to WT and EV cultures. Nude mice injected subcutaneously or intraperitoneally with clonal cell lines overexpressing OGFr had increased latencies to tumor formation, and the tumors were markedly decreased in volume/number, weight, and exhibited increases in OGFr
expression compared to mice injected with WT or EV cells. These data on the stable overexpression of OGFr indicate that OGFr is vital to cell replicative events in human ovarian cancer both *in vitro* and *in vivo*, and support treatments that amplify OGFr to decrease the growth of these neoplasias.

shRNA constructs were prepared to knockdown OGFr in SKOV-3 cells; 2 clonal lines were examined. OGFr protein expression was decreased up to 73% in clones compared to wild-type (WT) and empty vector (EV) controls; binding assays of clones revealed 50-55% decreases in binding capacity compared to controls. Growth and DNA synthesis were increased 33-146%, and doubling times decreased 29-35% compared to WT and EV cultures. Nude mice injected subcutaneously with cells underexpressing OGFr had an increased tumor incidence, decreased latency to tumor formation, and tumors were increased in volume and decreased in OGFr expression compared to WT and EV controls. OGF treatment in mice with WT or EV tumors, but not OGFr underexpressing tumors, markedly inhibited tumor volume, weight, and DNA synthesis. These data on the stable underexpression of OGFr demonstrate the critical nature of the OGF-OGFr axis in the progression of human ovarian cancer.

In summary, upregulation of the OGF-OGFr axis by treatment with exogenous OGF, pharmacologic upregulation of OGF and OGFr by short term opioid receptor antagonism with LDN, or stable molecular overexpression of OGFr, all inhibited ovarian cancer cell proliferation and tumor growth in a non-toxic manner. This pre-clinical data supports treatment modalities that amplify OGF/OGFr to decrease the growth of these neoplasias. The critical role of OGFr in the progression of this cancer was documented through experiments evaluating the repercussions of stably underexpressing OGFr, and will be important in understanding factors determining the success/failure of therapeutic modalities.
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List of Abbreviations

ANOVA analysis of variance

B<sub>max</sub> binding capacity

bp base pair

BrdU bromodeoxyuridine

CA-125 cancer antigen 125

CD10, NEP neutral endopeptidase

CD13, APN aminopeptidase N

CD4 cluster of differentiation 4

CD25 cluster of differentiation 25

CDK cyclin dependent kinase

cDNA complementary DNA

Ci curie

CKI cyclin dependent kinase inhibitor

CMV Cytomegalovirus

δ delta

DAMGO [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Glyol<sup>5</sup>]-enkephalin

DNA Deoxyribonucleic acid

dNTP Deoxynucleotide Triphosphate

DOR delta opioid receptor

DPDPE [D-Pen<sup>2,5</sup>]-enkephalin

EV empty vector

FCS fetal calf serum

FDA food and drug administration

fM femtomole

fmol femtomole

FOXP3 forkhead box P3

G<sub>1</sub> gap 1 phase of the cell cycle

G<sub>2</sub> gap 2 phase of the cell cycle

h hour

hr hour
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HDN</td>
<td>high dose naltrexone</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IGEPAL</td>
<td>octylphenoxypolyethoxyethanol</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>κ</td>
<td>kappa</td>
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<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>binding affinity</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
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<tr>
<td>KOR</td>
<td>kappa opioid receptor</td>
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<tr>
<td>l</td>
<td>length</td>
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<tr>
<td>LPS</td>
<td>lipopolysacharide</td>
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<td>LDN</td>
<td>low dose naltrexone</td>
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<td>μ</td>
<td>mu</td>
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<tr>
<td>mer</td>
<td>number of base pairs composing an oligonucleotide</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<td>μL</td>
<td>microliter</td>
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<td>μm</td>
<td>micrometer</td>
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<td>M</td>
<td>molar</td>
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<td>mg</td>
<td>milligram</td>
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<td>min</td>
<td>minutes</td>
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<td>mL</td>
<td>milliliter</td>
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<tr>
<td>mm</td>
<td>millimeter</td>
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<tr>
<td>mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>millimeter cubed</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>mmol</td>
<td>millimoles</td>
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<tr>
<td>MOR</td>
<td>mu opioid receptor</td>
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<tr>
<td>M phase</td>
<td>mitosis phase of the cell cycle</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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NAL  naloxone
Nal  naloxone
NK cell  natural killer cell
NLS  nuclear localization signals
nM  nanomolar
NTX  naltrexone
NIH  National Institutes of Health
OGF  opioid growth factor
OGFr  opioid growth factor receptor
P  probability
PBMC’s  peripheral blood mononuclear cells
PHA  phytohaemagglutinin
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PPE  preproenkephalin
RIPA  radioimmunoprecipitation assay
RNA  Ribonucleic acid
RT-PCR  reverse transcription polymerase chain reaction
S  synthesis phase of the cell cycle
s.c.  subcutaneous
SCCHN  squamous cell carcinoma of the head and neck
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE  standard error
s.e.m.  standard error of the mean
siRNA  small interfering RNA
shRNA  short hairpin RNA
taxol  paclitaxel
TGF-β  transforming growth factor beta
TUNEL  Terminal deoxynucleotidyl transferase dUTP nick end labeling
TIL’s  tumor-infiltrating lymphocytes
Tregs  T regulatory cells
<table>
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<tr>
<td>U</td>
<td>units</td>
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<tr>
<td>vol/vol</td>
<td>volume per volume</td>
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<tr>
<td>w</td>
<td>width</td>
</tr>
<tr>
<td>wt/wt</td>
<td>weight per weight</td>
</tr>
<tr>
<td>WT</td>
<td>wild type (untransfected)</td>
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Acknowledgements

I would like to thank the members of my thesis committee who played a guiding role in the design of this work. First, I would like to thank my thesis advisor, Dr. Patricia McLaughlin, for countless hours of mentorship throughout the years. The lessons I have learned from you will be invaluable in my future endeavors. Second, I would like to thank Dr. Ian Zagon. Your passion for science is inspiring, and I have learned so much from you about research design and writing manuscripts. To Dr. McLaughlin and Dr. Zagon, thank you for giving me the opportunity to work in your laboratory; it has been a pleasure. Next, many thanks to Dr. Robert Bonneau for your leadership and supervision on the immunology side projects I completed, that resulted in two publications. Thanks for allowing me to use your laboratory equipment to complete the work, and for taking the time to teach me so many techniques. To Dr. Ronald Wilson, thank you for sharing your animal expertise and for providing valuable insight into the animal models utilized. Finally, thank you to Dr. Michael Verderame, your expertise in molecular biology has been invaluable, and I have learned so much from you. This project was truly a collaborative effort, and I thank you all for your contribution and guidance.

In addition to my committee members, I have received help and support from many others. Thank you to the past and present members of the Zagon/McLaughlin lab, including Matt Klocek, Chris Pothering, Kristen Rahn, Anna Kober, Jessica Wentling, Cara Keiper, Jody Hankins, Fan Cheng, and Nancy Porterfield. Many thanks to the graduate program staff, especially Lori Coover, Karen Shields, Kathy Simon, and Dr. Henry Donahue. I would also like to thank my parents Don and Cathy Ertley, brother Daniel Ertley, and husband Patrick Donahue for their love and support throughout the years.
Chapter 1: Introduction
1. Chapter 1: Introduction
   1.1. Ovarian Cancer

   1.1.1. Disease Introduction, Etiology, and Classification

   Ovarian cancer is the leading cause of death from gynecological malignancies, and the 5th leading cause of cancer related mortality among women in the United States, with 21,880 new diagnoses and 13,850 deaths estimated in 2010 (1). Worldwide, it is estimated that 140,200 women die annually from ovarian cancer (2). The overall predicted lifetime risk of developing ovarian cancer is estimated at 1 in 70, or 1.43% (3).

   Ovarian cancer is a nonspecific term for a number of cancers that originate in the ovary, which is an organ that normally functions to produce ova and the steroid hormones, estrogen and progesterone. It is broadly accepted that the bulk of ovarian carcinomas (90%) are epithelial in origin, arising from ovarian epithelial cells of the surface and inclusion cyst epithelium (4-7), although recent work has suggested that fallopian tube epithelium may play a more important role than had been previously thought (8-10). The remaining ovarian cancers (10%) are believed to originate from stromal granulosa cells, theca cells, and germ cells (11).

   Epithelial ovarian cancers are classified into 5 histological subtypes: serous (30-70% of cases), endometrioid (10-20% of cases), mucinous (5-20% of cases), clear cell (3-10% of cases), and undifferentiated (<1% of cases) carcinomas, and the 5-year survival rates for these subtypes are 20–35%, 40–63%, 40–69%, 35–50%, and 11–29%, respectively (12-14). These subtypes differ with regard to risk factors, biological behavior, and treatment response.

   Tumorigenesis is a multi-step process that reflects a number of possible abnormal changes in cellular physiology, including a gain in self-sufficient growth signals, insensitivity to growth inhibitory signals, an ability to evade apoptosis, a potential for unlimited replication, a capacity for sustained angiogenesis, and ability for tissue invasion and metastasis (15). The currently held view of ovarian tumorigenesis is that carcinoma begins in the ovary, and then spreads by direct extension to the bladder, rectum, uterus, or peritoneum, lymphatic dissemination to the pelvic and/or para-aortic
lymph nodes, and/or dissemination through the entire abdomen by movement of ascites, peristalsis of the small intestines, or diaphragmatic respiratory motion (3, 7). In contrast to many other cancers, no anatomical barrier exists to block widespread metastasis of ovarian cancer throughout the peritoneal cavity (16).

Although the molecular events leading to the development of epithelial ovarian cancer are unknown, a number of hypotheses have been proposed. The first, the ‘incessant ovulation’ hypothesis, suggests that the risk of epithelial ovarian cancer increases with the number of ovulations (17). Each ovulation requires breakage of the ovarian follicle and repair of the ovarian surface, resulting in an increased number of cell divisions. Disorderly repair is believed to lead to the accumulation of a series of genetic alterations (16). Growth factors are thought to influence post-ovulatory repair, and dysregulation of growth factors may be involved in malignant transformation (18). A second hypothesis, the ‘pituitary gonadotropin’ hypothesis, suggests that postmenopausal elevations in gonadotropin act in concert with estrogen to stimulate the transformation of human ovarian surface epithelial cells (19). In a third hypothesis, chronic inflammation, caused by repeated ovulation, endometriosis, or pelvic inflammation, is believed to contribute to the development of ovarian cancer (20).

1.1.2. Risk Factors

Epidemiological studies have identified a number of risk factors including family history, nulliparity, delayed childbearing, early menarche and late menopause, increasing age, and residence in North America as being associated with an elevated risk for development of ovarian cancer (21). Rates of ovarian cancer are highest among white women in Europe and the United States, and lower in women from Central and South America (22). Although an identifiable genetic predisposition is present in only approximately 5% of affected women, a strong family history of ovarian or breast cancer is considered to be the most important risk factor (23).

1.1.3. Symptoms and Diagnosis

Early detection of ovarian cancer is hampered by the lack of appropriate tumor makers, and absence of clinically significant symptoms until the disease reaches an advanced stage (11). Although ovarian cancer has been termed the “silent killer”, more than 80% of patients with ovarian cancer have a spectrum of non-specific symptoms
during the early stages of disease pathogenesis that are shared by many more common gastrointestinal, genitourinary, and gynecological conditions (24, 25). This lack of a unique signature of symptoms likely accounts for late stage diagnosis of this disease. Symptoms of late advanced ovarian cancer are typically due to ascites; only when the ascetic fluid is detected by an ultrasound or a CT scan, or a mass is felt, will the diagnosis be considered, and diagnosis is made during exploratory surgery (24).

Serum cancer antigen 125 (CA-125) levels are elevated in more than 80% of patients with advanced epithelial ovarian cancer, but this measure alone is not sensitive or specific enough to be used diagnostically (23, 26, 27). Elevated CA-125 levels are associated with various conditions including pregnancy, endometriosis, adenomyosis, uterine fibroids, pelvic inflammatory disease, menstruation, benign cysts, or other malignant conditions such as pancreatic, breast, lung, gastric, and colon cancers (23, 28). However, a serum CA-125 level of more than 65 U/mL in a postmenopausal woman with an abdominal or pelvic mass raises the possibility of ovarian cancer, and should prompt consultation with a gynecologic oncologist (29). Although CA-125 levels are not usually helpful in preoperative evaluation, they are useful in assessing a patient’s response to postoperative chemotherapy, as well as in detecting early relapse in patients who have already received a diagnosis (30).

Despite ongoing efforts to develop effective screening strategies, only 20% of ovarian cancers are diagnosed in the early stages when they are limited to the ovaries (Stage 1), when up to 90% of patients can be cured using available therapies (31). After the disease has metastasized to the pelvic organs (stage 2), the abdomen (stage 3), or beyond the peritoneal cavity (stage 4), the cure rate decreases substantially (25, 31). Unfortunately, screening strategies to detect ovarian cancer in an earlier stage of the disease have failed to show any impact on survival; thus, optimizing treatment strategy remains the only way to improve the outcome of ovarian cancer patients (32).

1.1.4. Treatment of Epithelial Ovarian Cancer

Since Griffiths demonstrated the presence of an inverse relationship between overall survival and residual tumor size (33), aggressive cytoreductive surgery has played an important role in the initial management of ovarian cancer. Ovarian cancer is one of the few malignancies where surgery is performed to remove the bulk of a tumor
even though complete resection is impossible (25). Optimal debulking surgery consists of removal of the uterus, tubes, and ovaries as well as any large nodules of cancer (34). After surgery, almost all patients require chemotherapy. Intravenous administration of a combination of a platinum containing compound (carboplatin, cisplatin) plus a taxane containing compound (paclitaxel (taxol), docitaxel) is the current standard regimen for advanced ovarian cancer patients (23, 32, 35). Platinum analogues are the most active agents in this disease, mediating their effects through the formation of inter- and intra-strand cross-links with DNA (23), while taxanes exert their cytotoxic effects through a unique mechanism involving binding to and stabilization of the tubulin polymer (36).

Ovarian cancer is distinguished by initial sensitivity to chemotherapy with the majority (~75-80%) of ovarian cancers responding to first line platinum- and taxane-based chemotherapy (37); however, most patients (65%) subsequently relapse within 12-18 months (38, 39). Depending on the volume of residual disease following initial surgery, median progression free survival ranges from 16-21 months and median overall survival ranges from 24-60 months (39). Ultimately, nearly 60% of patients die within 5 years of diagnosis (39). Even with extensive surgical debulking and chemotherapy, the prognosis of late-stage ovarian cancer is dismal (1). Once ovarian cancer recurs all subsequent treatments are palliative (3). Major improvements in ovarian cancer treatment will likely require new molecularly targeted therapies that are based on exploitation of biological pathways (39). The availability of targeted agents to be added to chemotherapy, as well as a more profound knowledge of the complex biology of this disease, represent the most promising avenues to improve the prognosis of ovarian cancer patients (40, 41).

1.1.5. Cell Cycle Regulation

Dysregulation of cell proliferation is an integral component of the ovarian cancer phenotype (42). Regulatory mechanisms of the cell cycle include the cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CKI). Alteration of these systems results in uncontrolled cell proliferation, which is a distinctive feature of human cancers, including ovarian cancer. Upregulation of cyclin D1 or E1, E2F1, or cyclin dependent kinases (CDK), and downregulation of CKI’s (p16, p21 and p27) have been observed in a fraction of ovarian cancers (43, 44). The expression of p16 in particular seems to
relate to tumor grade and histologic type; however, some investigators report strong expression of p16 (45, 46), while others indicate loss of p16 expression (47) as being associated with higher tumor grades. Recently, a number of studies have indicated that decreased p16 expression is an unfavorable prognostic factor for patients with ovarian cancer, associated with overall decreased survival and resistance to chemotherapy (48, 49). As for p21 expression, 23% to 72% of epithelial ovarian cancers express this CKI (50-52); however, there appears to be an inverse correlation between p21 expression and tumor grade as well as proliferative index (50, 52). Low levels of p21 in patient samples are typically associated with poor prognosis (50, 53, 54). In addition to alterations in cell cycle checkpoints, a number of autocrine and paracrine growth factors have been shown to stimulate ovarian cancer proliferation, including epidermal growth factor, transforming growth factor α, amphiregulin, heregulin, vascular endothelial growth factor, insulin like growth factor 1, and interleukin 6 (25).

1.2. Models of Ovarian Cancer

1.2.1. History

Efforts to establish models to study epithelial ovarian tumorigenesis date back to the 1970’s, when investigators exposed rodent ovaries to carcinogens (55, 56). Although this method successfully generated tumors, the precise cellular origin and morphological features of these lesions were not well characterized (55, 56). Later in the 1980’s rat ovarian epithelial cells were established in vitro and ovarian cancer induced by transfecting these cells with a virus (57). In the 1990’s, studies by Godwin et al revealed that prolonged culture of primary rat ovarian epithelial cells in vitro, which mimics the rupture of the follicle during ovulation and subsequent repair processes, rendered these cells tumorigenic when injected into mice (58). In these late passaged cells, genomic analysis identified gross karyotypic instabilities (rearrangements, deletions, and gains) as being one of the earliest events predisposing rodent epithelial cells to transformation (58, 59). Later in 2000, Roby et al developed a model system where spontaneously transformed mouse ovarian epithelial cells formed tumors when injected in either immunocompromised or immunocompetent mice (60).
1.2.2. Limitations

Although nonhuman cell-based models have provided key insights about human ovarian tumorigenesis, biologic disparity between other species and humans complicates interpretation. For example, primary human ovarian surface epithelial cells are much more resistant to transformation than similar cells taken from mice and rats, and are not tumorigenic in mice (61, 62). In the 1970’s a number of cell lines of human ovarian cancer were established, most of which were derived from ascites or pleural effusions (63-65). Based on literature searches, two of the most common cell lines utilized both in vitro and in vivo for preclinical studies are the human epithelial ovarian cancer cell lines SKOV-3 and OVCAR-3. SKOV-3 was established in 1973 from the ascites of a 64 year old patient with ovarian adenocarcinoma (64), while OVCAR-3 was established in 1982 from the malignant ascites of a 60 year old patient with progressive adenocarcinoma of the ovary (66).

First reported in 1969 (67), xenografts of human cancer cells in immune-deficient mice have been widely used as a tool to both demonstrate the tumorigenicity of cells, as well as to test the efficacy of therapeutic compounds in vivo. Transplantation of human ovarian tumor xenografts into immunodeficient mice has offered useful information, but has limitations including incomplete recapitulation of tumor-host interactions, as well as the inability to replicate early stages of tumor development (7, 68). A criticism of many xenograft models is the relevance of the location of tumor formation and ability to parallel the human disease. To overcome this problem, many investigators utilize a combination of subcutaneous and intraperitoneal xenografts in testing the efficacy of therapeutic compounds for ovarian cancer (69-71). The intraperitoneal xenograft model of ovarian cancer, where human ovarian cancer cells are injected intraperitoneally and disseminated throughout the abdomen of mice is similar to human disease, with cell accumulation and tumor growth commonly occurring on the surface of the liver, stomach, and spleen, as well as adhering to the walls of the peritoneal cavity (72). Although considered a more clinically relevant model of ovarian cancer than the subcutaneous xenograft model, the major limitation of the intraperitoneal xenograft model is difficulty in monitoring disease formation and progression (72). Visual assessment of tumors in the intraperitoneal cavity is possible with magnetic resonance
imaging (73) or with cells that stably express green fluorescent protein (74); however, the equipment needed to visualize tumors by these methods is cost prohibitive. Furthermore, the location and availability of this equipment must also be considered, as immunodeficient mice must be transported from their protective environment for frequent assessment of tumor growth.

1.3. Opioids

1.3.1. Opioid Peptides

The medical and social importance of opiate and opiate-like compounds (collectively referred to as opioids) dates back thousands of years (75, 76). The discovery of opioid receptors in 1973 (77-79), and the discovery of endogenous opioids by Hughes and colleagues in 1975 (80, 81), stimulated an interest in elucidating the mechanism and function of opioids. Endogenous opioids are peptides that are produced and found throughout the body, and are known to be involved in a variety of processes including neurotransmission, modulation of neuroendocrine function, and growth regulation (82-84). Approximately 20 unique endogenous opioids have been identified, all of which are derived from three precursor proteins: pro-enkephalin, pro-opiomelanocortin, and pro-dynorphin (82, 85). Endogenously produced opioids are classified as enkephalins, endorphins, endomorphins, and dynorphins, which although differ in receptor affinity and action, have a high degree of sequence homology (83, 86). Endomorphins contain the amino acid sequence Tyr-Pro-Trp/Phe-Phe, while enkephalins, endorphins, and dynorphins contain the amino acid sequence Tyr-Gly-Gly-Phe (85, 86). Each endogenous opioid has a unique distribution in the body (85); the highest concentration of enkephalins, for example, is in the corpus striatum, with [Met\(^5\)]-enkephalin nearly 10-fold higher than [Leu\(^5\)]-enkephalin (87, 88). Immunohistochemical analysis of enkephalin distribution in mature and developing rat brains revealed higher levels in developing than adult brains (89). Enkephalins and endorphins are also found in a variety of human and animal tumors, including breast, prostate, ovarian, and liver cancers (90).

Expression levels of endogenous opioids are modulated under various physiological conditions, as well as in response to opioid receptor antagonism. The expression of β-endorphin, for example, markedly increases in response to strenuous
activity (91), as well as in response to formalin-induced pain and inflammation (92). Prodynorphin mRNA levels are increased in immune cells in response to chronic and acute inflammation (93), and both preproenkephalin (PPE) and preprodynorphin expression are increased following excitotoxic spinal cord injury (94). Opioid receptor antagonism has been shown to decrease dopamine release (95), which in turn alters expression of both dynorphin and enkephalin (96). Acute opioid receptor antagonism has also been shown to increase the expression of PPE as well as [Met$^5$]-enkephalin in developing rat brain (97), and plasma [Met$^5$]-enkephalin in mice with xenografts of colon cancer (98). Daily opioid receptor antagonism in children with autism was shown to dramatically increase plasma β-endorphin levels (99).

1.3.2. Opioid Receptors

There are three major classes of opioid receptors: mu (μ), delta (δ), and kappa (κ), which are widely distributed through the CNS, autonomic nervous system, peripheral nervous system, and various tissues and organs (83, 100, 101). Differential expression of these opioid receptors throughout the body indicates a unique and specific function for each receptor (83). The classic μ, δ, and κ opioid receptors are seven transmembrane G-protein coupled receptors located in the cell membrane, that function by converting extracellular signals into intracellular messages (85, 102). Each receptor has a differential affinity for endogenous and synthetic opioid peptides (85). Endomorphins have a high selectivity for μ opioid receptors, while enkephalins bind with the highest affinity to δ opioid receptors (85). In contrast, β-endorphin has equal affinity for μ and δ opioid receptors (85), and dynorphins bind to κ opioid receptors with the highest affinity (85). The classic opioid receptors are present in tumors originating from a variety of organs, including ovarian cancer (90).

A number of studies have demonstrated that opioid receptor profiles change with age, under various physiological conditions, and in response to opioid receptor antagonism. Receptor binding studies have indicated that μ, δ, and κ opioid receptor number is 2-, 7.8-, and 3.6-fold higher, respectively, in cerebellum from newborn humans compared to mature adults (103). In response to morphine, animals with peripheral inflammation showed increased nociception, indicating altered μ opioid receptor signaling under inflammatory conditions (93). Opioid receptor expression has
also been shown to be altered in response to opioid receptor antagonists. Receptor binding studies have indicated that δ and κ opioid receptor expression is significantly increased and decreased, respectively, in mice with xenografts of neuroblastoma in response to opioid receptor antagonism (104).

1.3.3. Opioid Receptor Antagonists

Naltrexone (NTX) and naloxone (Nal) are non-selective opioid receptor antagonists that are utilized to block the interaction of opioids and opioid receptors. Synthesized in 1960, Nal was unique from existing opioid receptor antagonists in that it did not produce dysphoric side effects (105). However, with a half-life of only 30 min [101], Nal could not be utilized as a treatment for opioid addicts because it did not provide a long lasting opioid receptor blockade. NTX was synthesized in 1963, and serves as a more potent and longer-lasting opioid receptor antagonist (105). The half life of NTX ranges from 8-10.3 h, depending on route of administration, and in contrast to Nal, is orally effective (105, 106). The FDA granted approval for NTX to be used in the treatment of alcohol and opiate addiction in 1994, and the patent for NTX expired in 1985 (107).

1.3.4. Growth Regulatory Function of Opioids

Although exogenous opioids, including morphine, methadone, and heroin have well known analgesic and behavioral effects, these compounds have also been reported to alter cell function in developing neural systems (108, 109). Exogenous opioids have been demonstrated to be antimitotic, cytotoxic, and damaging to chromosomes (110), retarding growth and development of many diverse organisms and tissue types (111-114). To determine whether exogenous opioids were also effective in inhibiting carcinogenic processes, Zagon et al evaluated the effects of chronic heroin, alone and in combination with the opioid receptor antagonist Nal in mice with neuroblastoma xenografts (115). An opioid receptor mediated inhibition in tumor growth and prolonged lifespan was observed in mice treated with heroin (115). In these studies, it was importantly noted that mice treated with Nal alone had significantly increased survival times compared to controls, with no associated toxicities noted with Nal’s use (116). The authors hypothesized that Nal could provide an over compensating release of
endogenous opioids, which in turn could act like heroin to inhibit cancer progression (115, 116).

Subsequent studies by Zagon et al evaluated whether the opioid receptor antagonist NTX, which is 8 times as active and 3 times as long acting as Nal (117), would have a similar antitumor effect in mice with neuroblastoma xenografts (118). It was discovered that a low dose of NTX (LDN, 0.1 mg/kg) inhibited tumor progression (decreased tumor incidence, delayed tumor incidence, increased survival), while a high dose of NTX (HDN, 10 mg/kg) accelerated tumor progression (decreased time to tumor formation, reduced survival) (118). These effects occurred with dosages of NTX that were 0.01 to 2% of the LD50 (570 mg/kg) (117). Studies were then undertaken to examine whether the dosage effects were related to the duration of opioid receptor blockade. Mice treated with LDN or HDN were challenged with morphine and tested on a hot plate (118, 119). Animals treated with morphine demonstrated analgesia when placed on a hot plate. NTX’s presence prevented the interaction of morphine with opioid receptors and the animals responded to the heat stimulus. It was demonstrated that LDN only blocked the opioid receptors for 4-6 h/day, while HDN invoked an opioid receptor blockade for at least 24 h (118). In subsequent studies it was demonstrated that the antitumor effects of LDN were dependent upon the duration of opioid receptor blockade and not dosage of NTX (119). These data elegantly demonstrated the relationship between tumor growth and the pharmacological action of an opioid receptor antagonist, revealing that endogenous opioid - opioid receptor interactions are crucial in modulating tumor response.

### 1.3.5. Opioid Peptide and Receptor Involved in Growth Regulation

Studies were next undertaken to determine the specific endogenous opioid peptide invoked by intermittent opioid receptor antagonism that was responsible for mediating the inhibitory actions on growth. Using a tissue culture system of murine neuroblastoma cells to assess the effects of opioid peptides and synthetic analogs related to pro-enkephalin A, pro-dynorphin, and pro-opiomelanocortin on cell proliferation, [Met5]-enkephalin, [Met5, Arg6, Phe7]-enkephalin, [Met5, Arg6, Gly7, Leu8]-enkephalin, and [Leu5]-enkephalin were found to exhibit inhibitory properties, with [Met5]-enkephalin being the most potent compound to influence cell replication (120).
Next, using mice transplanted with neuroblastoma cells to induce tumor formation, the effects of daily injections of [Met$^5$]-enkephalin or [Leu$^5$]-enkephalin on tumor progression was evaluated (121). In contrast to mice treated with [Leu$^5$]-enkephalin that did not differ in tumor progression from saline administered mice, animals treated with [Met$^5$]-enkephalin experienced delays in tumor formation and decreased mortality rates, suggesting that the key opioid regulating tumor growth is [Met$^5$]-enkephalin (121). Importantly, the inhibitory effects of [Met$^5$]-enkephalin were blocked by concomitant treatment with Nal, demonstrating that [Met$^5$]-enkephalin functions to inhibit tumor growth through interaction with an opioid receptor (121). To distinguish the role of [Met$^5$]-enkephalin as a growth factor from its role in neurotransmission, this peptide was termed the opioid growth factor (OGF).

Following the discovery that OGF was a native regulator of growth, the question arose as to which opioid receptor mediated its effects. A number of studies suggested the involvement of a non-classical opioid receptor. First, earlier experiments had demonstrated that administration of opioids selective for μ, δ, and κ opioid receptors did not alter cell proliferation (120). Additionally, xenografted animals treated with β-funaltrexone, a μ-specific opioid receptor antagonist, showed no changes in tumor incidence and tumor progression compared to saline administered controls (122). Further supporting the idea that growth regulation through modulation of endogenous opioid systems by NTX was not mediated by the μ opioid receptor, rats administered β-funaltrexone did not differ in brain weight or development relative to controls (123). Additionally, studies using synthetic ligands [D-Ala$^3$, D-Leu$^5$]-enkephalin and ethylketocyclazocine which are selective for δ and κ opioid receptors, respectively, found no influence of these compounds on tumor progression, suggesting that the inhibitory effects of OGF are not mediated by δ or κ opioid receptors (121). Finally, studies testing OGF’s action in COS-7 cells, a cell line derived from African green monkey kidney cells (124) that are devoid of classical opioid receptors (125-127), showed decreased cell proliferation, indicating the involvement of a non-classical opioid receptor capable of binding to OGF (128).

Receptor binding assays using $[^{3}H]$-[Met$^5$]-enkephalin in neuroblastoma homogenates taken from xenografted mice demonstrated the presence of a novel
opioid receptor with specific affinity for OGF (129). Displacement experiments in this study indicated that other ligands specific for the μ, δ, and κ opioid receptors were not highly competitive, having at least a 100-fold weaker affinity than OGF for the receptor (129). This novel binding site was saturable, stereoselective, and specific for drug displacement (129). These findings, coupled with previous immunohistochemical evidence of OGF in tumors and characterization of a function role of OGF, led to the discovery of the zeta (ζ) opioid receptor (129). This receptor that interacts with OGF to regulate cell proliferation was cloned, sequenced, and found to have no homology in nucleotide or amino acid sequence with the classical opioid receptors, as well as a unique cellular localization, being located on the nuclear membrane as opposed to the cell membrane like the classical opioid receptors (130). These key differences led to the proposal that the receptor be given a new name, the opioid growth factor receptor (OGFr) (130). This receptor has been sequenced in humans, rats, and mice, with substantial homology identified between these species, especially at the N terminal end of the 580, 634, and 677 amino acid rat, mouse, and human receptor, respectively (84). The gene for human OGFr is composed of seven exons and six introns, and using fluorescence in situ hybridization, was found to locate to chromosome 20q13.3 (84). Defects in this chromosomal location have been associated with a number of conditions, including autism, newborn epilepsy, and myeloproliferative disorders (84).

1.4. The OGF-OGFr Axis

1.4.1. Mechanism of Action

OGF is a native biological regulator of cell proliferation in cancer and normal cells (84, 131, 132) that is autocrine produced, and constitutively expressed and secreted (84, 120). The action of OGF is tonic, stereospecific, reversible, non cytotoxic and non apoptotic inducing, not associated with differentiative, migratory, invasive, or adhesive processes, and reliant on RNA and protein synthesis (84, 133-136). In tissue culture, OGF’s effects are independent of serum, anchorage independent, and occur at physiologically relevant concentrations (84, 137). The OGF-OGFr axis has been shown to inhibit cell proliferation at the G1/S phase of the cell cycle (138) through a unique cell signaling cascade that requires clathrin mediated endocytosis for OGF entry into cells (139), as well as nuclear localization of OGF and OGFr (140, 141). Once
OGF enters inside the cell, OGF binds to OGFr and the peptide-receptor complex is released at the outer nuclear envelope and moves to the perinuclear cytoplasm before undergoing nuclear transport that is dependent upon nuclear localization signals (NLS) encoded in OGFr (141), as well as transport by karyopherin β and Ran through the nuclear pore complex (140). In cells of squamous cell carcinoma of the head and neck (SCCHN), localization into the nucleus takes 8 h, and deletion of NLS 383-386 and NLS 456-460 on OGFr decreases nuclear receptor localization by 80% (141). The specific molecules in the nuclear pore complex that are essential for passage of OGF-OGFr, as well as the relationship between the OGF-OGFr complex and chromatin, require further clarification. However, studies have documented that p16 and/or p21 are upregulated by OGF and lead to a delay at the G1/S phase of the cell cycle (132, 142, 143).

### 1.4.2. Blockade of OGF-OGFr with NTX

In contrast to the inhibitory effects of OGF on cell proliferation, continuous opioid receptor blockade with NTX increases cell proliferation and increases the number of cells in the S and M phases of the cell cycle (138). As mentioned previously, NTX mediated blockade of endogenous opioids and opioid receptors is understood to result in a compensatory upregulation of opioid peptides and receptors to produce a supersensitive reaction in the case of LDN, where the upregulated peptides and receptors have the opportunity to interact. Both OGF and OGFr have been documented to be upregulated by NTX treatment. Administration of a dose of NTX causing complete opioid receptor blockade in rats causes a 67-183% increase in [Met⁵]-enkephalin levels from 5 min post-injection to 24 hours post-injection in the caudate putamen as compared to control rats (97). Following a dose of NTX producing intermittent opioid receptor blockade, levels of OGF in the cerebellum were increased 35% at 8-24 hours following NTX exposure (97). In an in vivo xenograft study, mice treated with LDN had a more than 2 fold increase in plasma levels of OGF relative to saline administered controls (98). With respect to the effects on OGFr, administration of NTX at a high dose caused an increase in the binding capacity of OGFr in the whole brain and cerebellum of rats, as well as an increase in the number of layers of germinal cells in the rat cerebellum as compared to vehicle-treated controls (144).
1.4.3. Modulation of OGF-OGFr Axis

An increase in OGF-OGFr activity in cells in vitro by the addition of exogenous OGF (133, 145-148), treatment with imidazoquinoline compounds such as imiquimod and resiquimod to upregulate OGFr (149), or transfection of sense cDNA for OGFr (150, 151), depresses cell proliferation. Similarly, upregulation of OGF-OGFr activity in vivo through exogenous administration of OGF (121, 152-154), intermittent opioid receptor antagonisms with LDN (98, 118, 119, 155), treatment with imiquimod (156), particle mediated gene transfer of OGFr using a gene gun (157), or stable molecular overexpression of OGFr (158, 159) inhibits cell proliferation.

Conversely, attenuation of the OGF-OGFr axis in cancer cells in vitro through disruption of OGF-OGFr interfacing by continuous exposure to NTX (131, 133, 145, 146), neutralization of OGF by antibodies to the peptide (131, 133), a decrease in OGFr by antisense cDNA or siRNA for OGFr (128, 131, 133), or a blockade of nuclear localization signaling, karyopherin β, Ran, or clathrin mediated endocytosis (139, 140, 160), stimulates cell proliferation. Similarly, blockade of OGF-OGFr interfacing by continuous opioid receptor blockade with HDN (118, 119, 155) in vivo accelerates cell replication.

1.4.4. The OGF-OGFr Axis and Cancer

The OGF-OGFr axis has been shown to regulate cell proliferation in a wide variety of human cancer cells, including cell lines representing neoplasms of SCCHN (SCC-1, CAL-27, SCC-4, SCC-9, SCC-25), esophagus (Flo-1), stomach (AGS), liver (SK-HEP-1, Hep G2), pancreas (Mia PaCa-2, BxPC-3, Capan-1, Capan-2, PANC-1), colon (HT-29, HCT 116, SW480, WiDR, COLO 205), lung (H 226, A549), melanoma (UACC-903, 1205-LU, G361), prostate (DU 145, PC3), uterus (MES-SA), breast (MDA-MB-231, MCF7), sarcoma (HT-1080, SK-ES-1), glioblastoma (U251), neuroblastoma (SK-N-SH, SK-N-AS), astrocytoma (SW 1088, U-87 MG), leukemia (K-562, HL-60), myeloma (U266), kidney (Caki-2, A498, SN12C, ACHN, Caki-1), and thyroid (KAT-18, KTC-1, WRO 82-1) (131, 145-148, 160, 161). Furthermore, in vivo tumor transplantation studies with pancreatic, colorectal, as well as SCCHN have documented that exogenous administration of OGF can decrease tumor incidence, delay tumor appearance, and retard the growth of these neoplasias (152-154, 158, 159, 162-165).
Moreover, using immunohistochemistry and/or receptor binding techniques, OGF and OGF\(_r\) have been documented in human surgical samples of pancreatic, colon, and thyroid cancer, as well as SCCHN (166-169).

A number of studies have indicated that OGF could be an ideal therapy for cancer when given in combination with standard of care treatments. Studies testing the effect of OGF in combination with taxol or gemcitabine in mice containing xenografts of SCCHN or pancreatic cancer, respectively, have shown that tumor progression is inhibited to a greater extent in mice receiving combination treatments, compared to mice receiving either OGF or chemotherapy alone (163, 165). Furthermore, OGF was additionally shown to reduce the toxic effects of chemotherapy without attenuating the anticancer action of chemotherapy in SCCHN (162). Phase I clinical trials have shown that OGF is a safe for administration in humans (170), and Phase II trials testing its efficacy are underway for its use in pancreatic cancer (171) and squamous cell carcinoma of the head and neck (personal communication, D. Goldenberg). Whether the OGF-OGFr pathway functions to regulate ovarian cancer and can be harnessed for the treatment of this deadly cancer, remains to be seen.

### 1.5. Connection Between Opioids and Ovarian Cancer

There are few papers relating endogenous opioids and ovarian cancer. Sporrong et al recorded immunoreactive enkephalin in primary ovarian carcinoids (172). Zagon et al noted both \(\delta\), and \(\kappa\) opioid receptors, but not \(\mu\) receptors, in an ovarian fibroma and stromal hyperplasia tumor, and detected both [Met\(^5\)]-enkephalin and \(\beta\)-endorphin in an ovarian fibroma (90). In 1987, Kikuchi et al reported that *in vitro* administration of Nal (30 to 120 micro M) for 72 h to KF human ovarian cancer cells inhibited cell proliferation in a dose dependent manner, while *in vivo* administration of Nal (3x/week with 2.5 or 5.0 mg/kg) initiated either one week prior to or 1 week after tumor inoculation reduced ovarian cancer tumor volume and increased mouse survival (173). Later in 1989, Kikuchi et al recorded that \(\beta\)-endorphin, \(\alpha\)-endorphin, and [Met\(^5\)]-enkephalin inhibited the growth of human serous cyoadenocarcinoma cells of the ovary *in vitro* in a dose-dependent manner that was partially reversed by Nal, and decreased protein and RNA synthesis, but not DNA synthesis (174). In a subsequent study, Kikuchi et al noted that \(\beta\)-endorphin (but not \(\alpha\)-endorphin or [Met\(^5\)]-enkephalin)
increased the lytic activity of spleen cells from mice with ovarian cancer, and concluded that opioid peptides play a role in immune surveillance mechanisms in ovarian cancer (175). Furthermore, Mollick et al reported antibodies to OGFr in the serum of 4 ovarian cancer patients, and suggested that OGFr may be useful target for vaccination (176). Finally, plasma β-endorphin levels in patients with ovarian carcinoma were found to be more than 2 fold higher than those of age-matched healthy women (177).

A number of studies have implicated one or more genes at 20q13 as having an important role in ovarian tumor growth and progression. Approximately 20% to 30% of ovarian tumors (preferentially stage III tumors) have an increase in the copy number of the 20q13 locus, and this variance in copy number is believed to have prognostic relevance (178-180). OGFr, interestingly, maps to this location at chromosome 20q13.3 (130).

1.6. Gap in Knowledge

It is known that endogenous opioids, specifically the OGF-OGFr axis, are involved in growth regulation of a variety of cancers and normal cells; however, the potential role this axis plays in the progression of ovarian cancer is unknown. This project was designed to determine whether the OGF-OGFr axis is involved in ovarian cancer cell proliferation and tumor growth, with the idea that upregulation of this axis could be translated into a successful therapy for ovarian cancer, to be administered either alone or in combination with standard of care treatments.

1.7. Hypothesis, Objectives, and Specific Aims

The central hypothesis for this thesis is that ovarian cancer progression is dependent on the interaction between OGF and OGFr, and that modulation of this axis may be utilized for therapeutic purposes. The objectives of this thesis are to establish whether pathways targeted by the OGF-OGFr axis are determinants in the progression of human ovarian cancer, and to determine whether human ovarian cancer can be better treated by combining agents that modulate the OGF-OGFr axis (OGF, LDN) with standard of care chemotherapeutic agents (paclitaxel, cisplatin).

NTX administration in vivo has been shown to upregulate OGFr (receptor binding by 45-66%) and to increase tissue OGF levels (4-8 fold). The rationale for the use of LDN is that upon clearance of LDN from OGFr, cell proliferation will be inhibited through
an upregulated OGF-OGFr interaction. The long term goal of this project is to improve ovarian cancer treatment. The hypothesis will be tested and objectives accomplished by pursuing the following specific aims:

**Specific Aim 1:** Ascertain the presence, specificity, ubiquity, function, and mechanism of the OGF-OGFr axis in human ovarian cancer cells *in vitro* using the cell lines OVCAR-3, SKOV-3, HEY, and CAOV-3. This aim will involve establishing whether increasing the activity of the OGF-OGFr axis by treating cells with OGF inhibits cell proliferation without altering apoptosis or necrosis, and determining whether the p16 and/or p21 cyclin dependent inhibitory pathways are involved in the inhibitory actions of the OGF-OGFr axis in human ovarian.

**Specific Aim 2:** Using an established subcutaneous SKOV-3 xenograft model of human ovarian cancer, ascertain whether OGF biotherapy inhibits tumor progression, and establish whether OGF can be combined with standard of care chemotherapies (paclitaxel, cisplatin) to elicit an additive inhibitory effect on ovarian cancer proliferation and tumor growth. This aim will involve establishing whether upregulation of the OGF-OGFr axis, by treating mice with OGF, alone and in combination with chemotherapies, alters ovarian tumor progression *in vivo*. Additionally, the mechanism(s) of tumor growth inhibition, including potential effects on programmed cell death, cell proliferation, and tumor angiogenesis will be ascertained.

**Specific Aim 3:** Establish a tissue culture model of LDN using short term opioid receptor antagonism, to evaluate the mechanisms by which LDN inhibits the growth of human ovarian cancer cells using the cell lines OVCAR-3 and SKOV-3. To date, short term opioid receptor blockade has been shown to modulate the OGF-OGFr axis *in vivo*. To determine whether the mechanism of LDN in inhibiting cell proliferation is by way of upregulation of the OGF-OGFr axis, a tissue culture model of short term opioid receptor blockade (LDN) will be established, and will involve receptor binding assays, northern and western blot analysis, RT-PCR, semiquantitative immunohistochemistry, radioimmunoassay, and evaluation of cell proliferation and growth effects *in vitro*.

**Specific Aim 4:** Using an established subcutaneous SKOV-3 xenograft model of human ovarian cancer, ascertain whether LDN biotherapy inhibits tumor progression, and establish whether LDN can be combined with standard of care chemotherapies
(paclitaxel, cisplatin) to elicit an additive inhibitory effect on ovarian cancer proliferation and tumor growth. This aim will involve establishing whether upregulation of the OGF-OGFr axis, by treating mice with LDN, alone and in combination with chemotherapies alters ovarian tumor progression \textit{in vivo}. Additionally, the mechanism(s) of tumor growth inhibition, including potential effects on programmed cell death, cell proliferation, and tumor angiogenesis will be ascertained.

**Specific Aim 5:** Utilizing a more clinically relevant \textit{in vivo} xenograft model of ovarian cancer where SKOV-3 cells are inoculated in the intraperitoneal cavity of nude mice, establish and compare OGF and LDN’s efficacy at inhibiting tumor progression. This aim will additionally involve establishing and comparing the mechanism(s) by which OGF and LDN regulate tumor growth in SKOV-3 intraperitoneal xenografts of human ovarian cancer.

**Specific Aim 6:** Ascertain \textit{in vitro} and \textit{in vivo} whether pathways targeted by the OGF-OGFr axis can be enhanced by stable molecular overexpression of OGFr in the human ovarian cancer cell line SKOV-3 to inhibit proliferation and tumor growth.

**Specific Aim 7:** Ascertain \textit{in vitro} and \textit{in vivo} the repercussions on ovarian cancer proliferation and tumor growth of stable underexpression of OGFr in SKOV-3 cells. Using tissue culture, as well as a subcutaneous xenograft model of human ovarian cancer, this aim will evaluate the efficacy of OGF treatment in the face of stable knockdown of OGFr.
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Chapter 2: OGF In Vitro

Rationale
The OGF-OGFr axis regulates proliferation of both normal cells as well as a variety of human cancer cell types. Having an interest in the pathogenesis of ovarian cancer, the goal of this first aim was to 1) establish that OGF and OGFr are present in human epithelial ovarian cancer cells, 2) determine whether the OGF-OGFr axis endogenously present in human ovarian cancer cells regulates ovarian cancer cell proliferation, 3) evaluate whether exogenous OGF inhibits ovarian cancer growth and characterize its inhibition, 4) ascertain whether OGF’s inhibitory effects in ovarian cancer work by way of OGFr, and 5) establish the mechanism by which OGF-OGFr inhibits human ovarian cancer number.
Cell Proliferation of Human Ovarian Cancer is Regulated by the Opioid Growth Factor - Opioid Growth Factor Receptor Axis

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*Am J Physiol Regul Integr Comp Physiol* 2009; 296:R1716-R1725

The Am Physiol Soc, used with permission
doi:10.1152/ajpregu.00075.2009
Abstract

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 in neoplasia. In this investigation we examined the relationship of the OGF-OGFr axis to ovarian cancer, and define its presence, function, and mechanisms. Using OVCAR-3 and SKOV-3 ovarian cancer cell lines, 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 repressive 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 and 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.
Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies (1), and is the 4th leading cause of cancer related mortality among women in the United States (2). Approximately 90% of primary ovarian cancers are epithelial tumors (3). 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 (4). 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 (1). Once ovarian cancer recurs, all subsequent treatments are palliative (4). 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 (1).

The opioid growth factor (OGF), chemically termed [Met\(^5\)]-enkephalin, is an endogenous opioid peptide that is an important regulator in the onset and progression of a variety of human cancers (5-10). OGF interacts with the OGF receptor (OGFr) to delay the G\(1/S\) interface of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways (11-13). Attenuation of the OGF-OGFr axis in cancer cells through: i) disruption of OGF-OGFr interfacing by continuous exposure to opioid antagonists (e.g., Naltrexone (NTX)) (5, 8, 14), ii) neutralization of OGF by antibodies to the peptide (5), or iii) a decrease in OGFr by antisense cDNA or siRNA for OGFr (15, 16), stimulates cell proliferation. An increase in OGF-OGFr activity in cancer cells by i) addition of exogenous OGF (5, 8-10), ii) treatment with imidazoquinoline compounds such as imiquimod and resiquimod (16), or iii) transfection of sense cDNA for OGFr (14, 17), depresses cell proliferation.

OGF has been detected by radioimmunoassay in surgical samples taken from human neoplasms of the ovary (18). The relationship of the OGF-OGFr axis to human ovarian cancer, however, is unknown. The present investigation explores the question of whether the OGF-OGFr axis is present and functions in human ovarian neoplasia and the mechanism(s) underlying these pathways.
Materials and Methods

Cell Culture

Human ovarian cancer cell lines, OVCAR-3 (19), SKOV-3 (20), CAOV-3 (20) and SW626 (21) were obtained from the American Type Culture Collection (Manassas, VA), and HEY (22) was a gift from Dr. Leslie Parent (The M.S. Hershey Medical Center).

OVCAR-3 cells were maintained in RPMI 1640 complete medium that was modified to contain 10 mM HEPES, 1 mM sodium pyruvate, 4.5g/L glucose, 1.5g/L sodium bicarbonate, 0.01 mg/mL bovine insulin, whereas the SKOV-3 cell line was grown in McCoy’s 5a Medium containing 1.5mM L-glutamine and 2.2g/L sodium bicarbonate; both media were supplemented with 10% fetal bovine serum. CAOV-3 and HEY cells were cultured in Dulbecco’s medium and SW626 cells were grown in Leibovits L-15 medium; both media were supplemented with 10% fetal calf serum (FCS). All cells except for SW626 were grown in a humidified atmosphere of 5% CO_2 /95% air at 37^0C; SW626 was maintained in a humidified atmosphere of 100% air. All media contained antibiotics (5,000 Units/mL penicillin, 5 μg/mL streptomycin, and 10 mg/mL neomycin). For some experiments, cultures of OVCAR-3 cells were maintained in a series of decreasing concentrations of FCS with no insulin in the media, with serum reduced over 4 weeks from 10% to 1.5%.

Immunohistochemistry

Log-phase OVCAR-3 and SKOV-3 cells were plated onto 22 mm round coverglasses, and 72 h later fixed and stained with anti-OGF and -OGFr antibodies according to published procedures (8-10, 23, 24). Polyclonal antibodies to OGF and OGFr were generated in the laboratory and have been fully characterized (25). Controls included cells incubated only with secondary antibodies. At least 3 coverglasses were examined.

OGFr Binding Assays

Receptor binding assays for OGFr were performed using log-phase cells (14, 16, 17) and custom synthesized [^3H]-[Met^5]-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; Co172); pre-immune rabbit serum (1:200) served as a control. Serum and media were changed daily, and cells counted after 72 h of treatment. Cell viability was determined, with 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’-uagaacucagguuuggcg-3’; sense, 5’-cgccaaaccugaguuucua-3’) was designed and obtained as a ready-annealed, purified duplex probe from Ambion (Austin, TX). 5 x 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 was added. At 72 h, cells were collected and either counted or harvested for RNA for Northern blot 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 re-probed with radiolabeled GAPDH and optical density of each band was determined and analyzed by QuickOne (Bio-Rad Laboratories). Each
value was normalized to GAPDH from the same blot. Means and SE were determined from at least 2 independent experiments.

To evaluate the level of OGFr protein knockdown, semiquantitative immunohistochemistry was utilized on a subset of cultures that were seeded and transfected with siRNA’s on round coverglasses. For quantification of OGFr protein levels, images were taken at 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 coverglasses/group.

**Mechanisms of OGF-Modulated Growth Inhibition**

The effect of OGF on DNA synthesis (BrdU incorporation), apoptosis (TUNEL), and necrosis (TUNEL and trypan blue) of human ovarian cancer cells were evaluated. Cells were seeded onto 22 mm diameter coverglasses placed in 6-well plates (5 x 10⁴ 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 x 10⁵) were synchronized with 50 nM nocodazole (Sigma-Aldrich) 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 10⁻⁶ 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 (1x PBS, 10 μM IGEPAL, 1 mg/mL SDS, 5 mg/mL deoxycholic acid), containing protease and phosphatase inhibitors (2 μg/mL aprotinin, 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: p21 (BD PharMingen, San Diego, CA), p16 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Clone AC-15,
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; 1:5000), and developed using a chemiluminescence 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 (1:5000).

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 2 independent experiments.

**siRNA Knockdown of p16 and p21**

Log phase cells were transfected with 20 nM concentrations of p16 and p21 siRNA’s (Santa Cruz Biotechnology) or scrambled siRNA’s (Ambion) with Oligofectamine reagent (Invitrogen) in serum and antibiotic free media for 4 hr at 37°C before the addition of OGF (10⁻⁶ 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 growth curves or for Western blotting 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 1000 cells/treatment on at least 3 coverglasses/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: [Met\textsuperscript{5}]-enkephalin, [Leu\textsuperscript{5}]-enkephalin, [D-Pen\textsuperscript{2,5}]-enkephalin (DPDPE), [D-Ala\textsuperscript{2},MePhe\textsuperscript{4},Glyol\textsuperscript{5}]-enkephalin (DAMGO), β endorphin, naltrexone (NTX), naloxone (NAL), dynorphin A1-8, morphine sulfate, endomorphin 1, endomorphin 2, Sigma (St. Louis, MO); U69,583, Upjohn Diagnostics (Kalamazoo, MI).

Statistical Analysis

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

OGF and OGFr are Present in Human Ovarian Cancer Cells

Immunoreactive OGF and OGFr were localized to the cytoplasm of OVCAR-3 and SKOV-3 cells, with speckling of immunoreactivity noted in the nucleus (Figure 2.1A). 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 (Figure 2.1B). The binding capacities ($B_{\text{max}}$) for OVCAR-3 and SKOV-3 were $4.5 \pm 0.5$ and $3.9 \pm 0.2$ fmol/mg protein, respectively, whereas the binding affinities ($K_d$) for OVCAR-3 and SKOV-3 were $4.2 \pm 1.0$ and $6.2 \pm 1.9$ 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 (Figure 2.2A). 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 to control cultures (Figure 2.2B).

To determine whether OGF activity was mediated by an opioid receptor, cells were grown in the presence of OGF and the short acting non selective opioid receptor antagonist naloxone (Nal) at a concentration of Nal that did not influence cell proliferation (Figure 2.2C). Cell number was reduced by 30% in both OVCAR-3 and SKOV-3 cultures treated with $10^{-6}$ M OGF compared to vehicle-treated cultures; cultures receiving both OGF and Nal, or Nal alone, returned to 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 was removed at 72 h, and fresh media added with no OGF (OGF-Co); some cultures continued to receive a daily change of media and OGF (OGF-OGF). At 24 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) (Figure 2.2D).

To examine whether there were confounding variables introduced by growing cells in 10% serum, OVCAR-3 cells were adapted over a 4 week 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).

**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 (Figure 2.3A). 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 (Figure 2.3A).

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 pre-immune serum (IgG) had a similar number of cells (Figure 2.3B).

**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 evaluated at the molecular level using siRNA technology. OGFr siRNA transfected OVCAR-3 and SKOV-3 cells had less than 70% and 53%, respectively, OGFr mRNA levels relative to cells not transfected (Figure 2.4A). Cells exposed to scrambled siRNA were comparable in OGFr mRNA levels to untransfected cells.
Semiquantitative immunohistochemistry revealed that OGFr siRNA transfected OVCAR-3 and SKOV-3 cells have approximately 75% of OGFr protein levels than cells not transfected (Figure 2.4B). OVCAR-3 and SKOV-3 cells transfected with OGFr siRNA had 31% and 23%, respectively, more cells than cultures that were not transfected (Figure 2.4C). The addition of exogenous OGF or NTX had no inhibitory or stimulatory, respectively, effects on cells transfected with OGFr siRNA compared to cultures treated with OGFr siRNA and sterile water.

OGF Alters DNA Synthesis but Not Apoptosis or Necrosis

To evaluate the mechanism by which OGF inhibits human ovarian cancer cell growth, DNA synthesis of OVCAR-3 and SKOV-3 cultures exposed to OGF, NTX or sterile water was measured (Figure 2.5A-C). The proportion of BrdU labeled cells in OVCAR-3 and SKOV-3 cultures exposed to OGF for 3 h was decreased by approximately 40% compared to cultures receiving sterile water, whereas cells given NTX increased by 43% and 31%, respectively, from cultures receiving sterile water.

Examination of apoptosis or necrosis in OVCAR-3 and SKOV-3 cells treated with OGF or NTX for 72 h revealed less than 0.1% positive cells for apoptosis or necrosis, and these data were comparable to that obtained with cells subjected to sterile water.

OGF’s Inhibitory Effects, 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, puromycin, cyclohexamide, or actinomycin D (Figure 2.5A-C). In contrast, NTX’s accelerating effects on cell proliferation in these ovarian cancer cells persisted in the presence of puromycin (Figure 2.5A), cyclohexamide (Figure 2.5B), or actinomycin D (Figure 2.5C).

p16/p21 is Required for OGF-Induced Growth Inhibition

To evaluate whether the mechanism of OGF activity is 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 in comparison to vehicle treated cultures by 2.6-fold following 1 h of OGF-treatment, and 4.1-fold after 9 h of OGF exposure (Figure 2.6A,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 (Figure 2.6A, 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 (Figure 2.6C). 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 (Figure 2.6D). For SKOV-3 cells, p21, but not p16, was required to induce OGF inhibitory action (Figure 2.6D). 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 to cells subjected to sterile water. SKOV-3 cells transfected with scrambled siRNA or p16 siRNA and exposed to OGF (10^{-6} M) for 72 h had reductions in growth of 40-50% in contrast to cells treated with sterile water.

The OGF-OGFr Axis is Present and Functions in a Variety of Ovarian Cancer Cells

The ubiquity of OGF’s inhibitory action in human ovarian cancer cells was examined in three additional cell lines, SW626, CAOV-3, and HEY (data not shown). 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.
Discussion

A previous study in our laboratory detected OGF by radioimmunoassay in surgical specimens of human ovarian neoplasms (18). This observation prompted two questions: i) Does the OGF-OGFr axis exist in human ovarian cancer? and ii) 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 opioid receptors, 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 5 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, upsetting the delicate balance in regulating cell number by peptide activity.

An extensive literature shows that OGF interacts with OGFr to regulate cell proliferation (5, 6, 8-14, 16, 17, 26-28), implying that effects on the control of cell number by 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 [Met\(^{5}\)]-enkephalin and this opioid peptide is known to bind to classical opioid receptors such as µ, δ, and κ as well (29). In order 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 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 transfected cultures given vehicle or 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 (24). 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 (25). 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, 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 (30). However, DNA synthesis in cultures treated with OGF exhibited a marked diminishment from control levels, whereas blockade of the OGF-OGFr interaction by NTX elevated DNA synthesis. Consistent with previous studies, these data indicate that the mechanism of OGF involves regulation of cell proliferation (8-14, 16, 17).

The effect of OGF and NTX on cell proliferation begs 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 and 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 (11-13), suggesting that cyclin-dependent kinase inhibitory (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 (13), 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. Similarly to
the finding in pancreatic cancer cells (12), ovarian cancer cells lacking p16, relied on the p21 CKI pathway to exert OGF’s action.

These results reveal for the first time, the presence and 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. Preclinical experiments are needed to establish whether this agent can alter the course of these lethal neoplasias.
Figure 2.1. The presence and distribution of OGF and OGFr in human ovarian cancer cells. (A) Photomicrographs of log-phase OVCAR-3 and SKOV-3 cells visualized with differential interference (DIC) or immunohistochemistry of samples stained with polyclonal, ammonium-sulfate purified antibodies (1:100) to [Met$^5$]-enkephalin (OGF) or OGFr. Rhodamine-conjugated IgG (1:1000) 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 $[^3]$H-[Met$^5$]-enkephalin to nuclear homogenates of OVCAR-3 and SKOV-3 cells. Mean ± SE binding affinity ($K_d$) and maximal binding capacity ($B_{max}$) from at least 4 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.
Figure 2.2. 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/well 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 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. Control cultures received sterile water throughout the 120 h. Compounds and media were replaced daily. For all experiments, data represent 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, or ***p<0.001.
Figure 2.3. OGF is the specific opioid peptide involved in the growth inhibition of human ovarian cancer cells. (A) The 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), pre-immune serum (IgG), or with 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 2 aliquots/well from at least 2 wells/group. Significantly different from respective controls at ***$p<0.001$. 

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**A**

**B**
**Figure 2.4.** 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 h after transfection, cells were harvested and RNA isolated. Data (percent of OGFr/GAPDH ratio) represent mean ± SE for 2 blots from independent experiments. Significantly different from non-transfected cultures at p<0.05 (*). (B) Photomicrographs of log phase OVCAR-3 and SKOV-3 cells stained with a polyclonal antibody to OGFr (B0344) 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 same exposure time. Quantitative measurement of the OGFr immunohistochemistry demonstrating 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 mean ± SE. Significantly different from non-transfected 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 represent mean ± SE cell counts for at least 2 aliquots/well and least 2 wells/treatment. Significantly different at *p<0.05 or ***p<0.001 from cultures that were not transfected.
Figure 2.5. OGF’s inhibitory effects on DNA synthesis, and its dependence on RNA and protein synthesis. NTX’s stimulatory effects on DNA synthesis are independent of RNA and protein synthesis. OVCAR-3 and SKOV-3 cells were seeded on 22 mm coverglasses and treated for 3 h with $10^{-6}$ M concentrations of OGF, NTX or an equivalent volume of sterile water, 5 µg/ml concentrations of puromycin (A), cyclohexamide (B), or Actinomycin D (C), and 30 µM BrdU. Data represent the percent BrdU positive cells (mean ± SE). Significantly different from water treated cells at *p<0.05, **p<0.01 or ***p<0.001. Significantly different from OGF treated cultures at ^p<0.05, ^^p<0.01 or ^^^p<0.001. Significantly different from cells treated with water and puromycin, cyclohexamide, or actinomycin D at +p<0.05, ++p<0.01 or +++p<0.001.
Figure 2.6. 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 1,2,3,4,5,6,9, 15, 18, or 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 0 h. The p16 and p21 level was significantly elevated from water treated cells at time 0 at $^*p<0.05$, $^{**}p<0.01$, or $^{***}p<0.001$. (C) OVCAR-3 and SKOV-3 cells were transfected for 24 h with p16 or p21 siRNA and total proteins were isolated 48 h after transfection and 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 represent mean ± 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.
References


Chapter 3: OGF *In Vivo* and in Combination with Chemotherapy

Rationale
The OGF-OGFr axis has been shown to be present and function to inhibit epithelial ovarian cancer cell proliferation *in vitro*. Whether this axis is present and functions *in vivo* to regulate ovarian tumor growth needs to be addressed. Based on the knowledge that the OGF-OGFr axis targets cell proliferation without inducing apoptosis, we examined whether this non-toxic biotherapy can be utilized in combination with the standard of care chemotherapies to provide an additive inhibitory effect for the treatment of ovarian cancer. Using an established subcutaneous xenograft model where tumor growth can easily be tracked over time, the goal of this study was to ascertain whether OGF biotherapy, administered alone and in combination with the standard of care chemotherapies taxol or cisplatin, regulates cell proliferation and tumor growth of ovarian cancer, as well as determine the mechanism of OGF’s action alone and in combination treatments.
The Opioid Growth Factor Inhibits Established Ovarian Cancer in Nude Mice and can be Combined With Taxol or Cisplatin to Enhance Growth Inhibition

Accepted for publication in *Journal of Cancer Therapy*

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Abstract

Ovarian cancer is the 5th leading cause of cancer-related mortality in women. Seventy-five percent of ovarian cancer patients present in advanced stages, and receive cytoreductive surgery and adjuvant chemotherapy. However, within 2 years 65% of these patients relapse and thereafter only receive palliative care. Novel therapies based on the biology of these cancers are urgently needed. The opioid growth factor (OGF)- OGF receptor (OGFr) axis is an endogenous opioid system known to inhibit proliferation of human ovarian cancer cells in tissue culture, but does not affect cell survival. The present study determined whether OGF in combination with standard of care chemotherapy, provides an inhibitory effect on the growth of human ovarian cancer cells in vitro. In addition, this investigation assessed whether OGF biotherapy, alone or in combination with taxol or cisplatin, inhibits tumor growth in mice with xenografts of ovarian cancer. The combination of OGF (10^{-6} M) with taxol (10^{-9} M or 10^{-10} M) or cisplatin (0.01 μg/ml or 0.001 μg/ml) markedly reduced cell number and DNA synthesis in vitro to a greater extent than individual compounds. OGF, but not taxol or cisplatin, altered growth in an opioid receptor mediated and reversible manner. Female nu/nu mice inoculated subcutaneously with SKOV-3 cells, and treated with OGF (10 mg/kg) for 5 weeks commencing at the time tumors became measurable, had tumor volumes and weight that were reduced by up to 50% from animals receiving saline. The combination of OGF with taxol (3 mg/kg, weekly) or cisplatin (4 mg/kg, weekly for 2 weeks) for 37 days reduced tumor volumes and weight in contrast to mice receiving individual agents alone. Moreover, OGF treatment in mice receiving cisplatin provided protection against the weight loss associated with cisplatin alone. All treatments suppressed DNA synthesis and angiogenesis, but exposure to taxol or cisplatin, but not OGF, induced apoptosis. Additive inhibitory effects on DNA synthesis and angiogenesis were recorded in animals treated with both OGF and taxol, or OGF and cisplatin, in comparison to individual compounds alone. OGF and OGFr were detected in tumor tissue; however OGFr expression was reduced 51-81% by OGF treatment. This preclinical evidence demonstrates that OGF biotherapy markedly inhibits ovarian tumorigenesis in a non-toxic manner, and can be combined with taxol or cisplatin to provide an enhanced therapeutic benefit.
Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies [1], resulting in an estimated 140,200 deaths worldwide annually [2]. Approximately 90% of ovarian cancers are epithelial in origin [3], and the most common presentation (75%) is in the advanced stages (stage III/IV). Cytoreductive surgery and adjuvant chemotherapy with taxol and a platinum containing compound serve as the standard of care [4]. Although initial clinical response is excellent [4], 65% of patients relapse within 2 years and thereafter only receive palliative care [1]. Major improvements in the treatment of ovarian cancer patients will require novel therapies that capitalize on biological pathways [1].

Dysregulation of the cell cycle is an integral component of ovarian cancer [5]. One native biological regulator of cell replication in normal cells and a variety of cancers, including ovarian cancer, is the opioid growth factor (OGF) and its receptor, OGFr [6-11]. OGF is a constitutively active native opioid peptide, chemically known as [Met$^5$]-enkephalin, that is autocrine produced and secreted, and interacts with OGFr to delay the G$_{1}$/S interface of the cell cycle without affecting cell survival by modulating cyclin-dependent kinase inhibitory (CKI) pathways [11-15]. Although OGFr pharmacologically resembles classical opioid receptors (recognizes opioids, naloxone (Nal) reversibility, stereospecificity), it shares no sequence homology, has a different cellular localization (detected in the outer nuclear envelope, nucleus, and perinuclear cytoplasm [6, 16-19]), and undergoes trafficking into the nucleus in a process that requires nuclear localization signals and transport by karyopherin $\beta$ and Ran [17, 20].

The OGF-OGFr axis has been shown to be present and involved in the regulation of human ovarian cancer cell proliferation in a tissue culture model [11, 21]. OGFr RNA, protein, and binding activity have been documented in ovarian cancer cells in vitro [11], and OGF has been detected by radioimmunoassay in surgical samples taken from human ovarian neoplasms [22]. Moreover, an increase in OGF-OGFr activity in human ovarian cancer cells in tissue culture by the addition of exogenous OGF has been shown to markedly suppress cell proliferation in a non-toxic manner by targeting the CKI pathways [11, 21]. The present investigation explores whether OGF biotherapy 1) modulates human ovarian tumorigenesis in mice with established
subcutaneous xenografts, and 2) can be combined with standard of care chemotherapies (taxol, cisplatin) to elicit an additive inhibitory effect on cell proliferation and tumorigenesis. These results demonstrate that OGF inhibits ovarian cancer *in vivo*, and can be combined with taxol or cisplatin for improved efficacy. Moreover, our data suggest that the toxic effects associated with chemotherapeutic agents utilized in the treatment of ovarian cancer can be alleviated by combining these drugs with OGF. Thus, the use of both chemotherapy and OGF biotherapy may provide a novel strategy in the treatment of this deadly disease.
Material and Methods

Cell Culture

The human ovarian cancer cell line SKOV-3 [23], obtained from the American Type Culture Collection (Manassas, VA), was grown in a humidified atmosphere of 5% CO\textsubscript{2}/95% air at 37\textdegree C in RPMI medium supplemented with 1.2% sodium bicarbonate, 10% fetal calf serum, and antibiotics (5,000 units/ml penicillin, 5 µg/ml streptomycin, and 10 mg/ml neomycin).

Growth Assays

Cells were plated and counted 24 h later (time 0) to determine seeding efficiency. Compounds or vehicle were added at time 0; media and compounds were replaced daily unless otherwise indicated. Taxol was dissolved in DMSO (10\textsuperscript{-2} M) and further diluted in sterile water; all other compounds were prepared in sterile water, and dilutions represent final concentrations. An equivalent volume of vehicle was added to control (Co) wells. Cells were harvested at designated times, stained with trypan blue, and counted with a hemacytometer. At least 2 aliquots/well and 2 wells/treatment/time point were sampled.

Animals, Tumor Cell Implantation, and Tumor Growth

Four week-old athymic nu/nu female mice, purchased from The Charles River Laboratory (Wilmington, MA), were housed in pathogen-free isolator ventilated cages in a controlled-temperature room (22-25\textdegree C) with a 12-12 h light/dark cycle (lights on 0700-1900). Sterile rodent diet (Teklad, Indianapolis, IN) and water were available ad libitum. All procedures were approved by the IACUC Committee of The Pennsylvania State University College of Medicine, and conformed to the guidelines established by the NIH. Following a 48 h acclimation period, unanaesthetized mice were injected subcutaneously (s.c.) with SKOV-3 cells (4 x 10\textsuperscript{6}/mouse) into the right scapula region. Mice were weighed 3x/week, observed daily for initial appearance of tumors, and tumors were measured 3 times/week using vernier calipers. Volume was calculated using the formula \( V = \frac{l \times w^2 \times \pi}{6} \) where length (l) is the longest dimension, and width (w) is the dimension perpendicular to the length [24].
Drug Treatment

Beginning on the day tumors became visible (day 0), six groups of mice (n = 12) were randomly assigned to receive intraperitoneal (i.p.) injections of OGF (10 mg/kg, daily), taxol (3 mg/kg, days 0, 7, 14, 21, 28, and 35), cisplatin (4 mg/kg, days 0 and 7), OGF (10 mg/kg, daily) and taxol (3 mg/kg, days 0, 7, 14, 21, 28, and 35), OGF (10 mg/kg, daily) and cisplatin (4 mg/kg, days 0 and 7), or an equivalent volume of saline (daily). These dosages and regimens were selected based on published reports [25-29]. To ensure that all mice received an equivalent number of injections, mice not assigned to receive treatment on a given day were injected with saline. In groups receiving combined therapy, OGF was administered first. Taxol was dissolved in DMSO (10^{-2} M) and further diluted in saline, while OGF and cisplatin were dissolved in saline; all drugs were prepared weekly.

Termination Day Measurements

According to the IACUC guidelines, the study was terminated when tumors became ulcerated or grew to 2 cm in diameter. All mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and cervical dislocation 37 days following initiation of treatments. For examination of DNA synthesis rates in tumors, a subset of mice from each group were injected i.p. twice with 100 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO) at 6 and 3 h prior to euthanasia. Tumors and spleens were removed and weighed, and the lymph nodes, liver, and spleen examined for metastases. Tumor tissues were assessed for expression of OGF and OGFr, cell survival, angiogenesis, and DNA synthesis.

Semiquantitative Immunohistochemistry

Immunohistochemistry was utilized to evaluate the presence and relative levels of OGF and OGFr in tumor tissue following published procedures [11]. Tumors were excised, frozen in chilled isopentane, sectioned at 10 μm, fixed, permeabilized, and stained with antibodies to OGF and OGFr that were generated in our laboratory [30]. Images were taken at the same exposure time with care not to photobleach the preparations. A random sample of at least 10 fields/section, 2 sections/tumor, and 2 tumors/group were evaluated. Controls were incubated with secondary antibodies only.
Protein Isolation and Western Blotting

Expression of OGFr was evaluated in tumors by Western blotting following published procedures [11]. Briefly, tissue was homogenized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN). Protein (60 μg) was subjected to 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to OGFr (1:200). Optical densities were normalized to β-actin (1:5000, Sigma-Aldrich), and the percent change in expression was calculated by dividing the normalized values of experimental samples to that of saline controls. Means and SE were determined from 2 independent experiments.

OGFr Binding Assays

Tumors were assayed for OGFr binding using custom synthesized [³H]-[Met⁵]-enkephalin (Perkin Elmer, Waltham, MA; 52.7 Ci/mmol) following published procedures [11, 30, 31]. Saturation binding isotherms were generated using GraphPad Prism software (La Jolla, CA), and independent assays were performed at least 3 times.

Mechanism of Growth Inhibition: DNA synthesis, Angiogenesis, Apoptosis and Necrosis

DNA Cells were assayed for DNA synthesis, necrosis, and apoptosis, whereas tumor tissue was evaluated for DNA synthesis, apoptosis, and angiogenesis. To measure DNA synthesis, cells were treated with 30 μM BrdU for 3 h prior to fixation, while tumors from mice receiving BrdU on the day of sacrifice were fixed in formalin overnight, processed in paraffin, and sectioned at 10 μm. Preparations were processed with antibodies to BrdU (1:200, Invitrogen, Carlsbad, CA) [11, 31, 32] to assess DNA synthesis, stained with Hematoxylin/Eosin [33, 34] to evaluate endothelial cell-lined vessels containing red blood cells, or processed for TUNEL according to the manufacturer’s instruction to measure apoptosis (Trevigen, Gaithersburg, MD). For cells in tissue culture, the proportion of BrdU or TUNEL positive cells was determined for at least 500 cells on 2 coverglasses/treatment group. For tumors, the proportion of BrdU positive cells, number of TUNEL positive cells, and blood vessel density were determined from at least 10 random fields around the periphery of each tumor, with at least 2 sections/tumor, and 2 tumors/treatment group evaluated. BrdU and TUNEL
positive cells were counted in a 0.003 mm$^2$ area, while blood vessel density was determined in a 0.16 mm$^2$ area.

**Chemicals**

OGF and Nal were obtained from Sigma-Aldrich. Cisplatin was purchased from Alexis Biochemicals (Lausen, Switzerland), and taxol was obtained from Toronto Research Chemicals (North York, ON).

**Statistical Analysis**

All data were analyzed using one way analysis of variance (ANOVA), with subsequent comparisons made using Newman–Keuls tests (Graph Pad Prism Software). In some cases, data were evaluated with unpaired $t$-tests; $p$ values less than 0.05 were considered significant.
Results
Combination of OGF with Taxol or Cisplatin Provides an Additive Inhibitory Effect on Cell Number: *In Vitro* Studies

To establish the efficacy of the combination of OGF with taxol on the growth of human ovarian cancer cells, SKOV-3 cultures were treated with OGF (10^-6 M, a dosage known to inhibit cell proliferation [11, 21]), taxol (10^-9 M or 10^-10 M, dosages selected because preliminary experiments revealed no logarithmic growth at higher concentrations), taxol and OGF, or an equivalent volume of sterile water and monitored for 120 h. Relative to sterile water treated controls, cell number was reduced in cultures exposed to OGF (17-36%), taxol at 10^-9 and 10^-10 M (26-47%), and OGF and taxol at 10^-9 and 10^-10 M (24-61%) (Figure 3.1A). In cultures receiving both OGF and taxol (at either concentration), cell number was reduced 16-48% from 72 h to 120 h compared to cells exposed to OGF alone. In comparison to cells treated with taxol alone (at either concentration), cell number in cultures treated with both OGF and taxol (at either concentration) was reduced 15-28% at 96 h and 120 h (Figure 3.1A). At all time points evaluated, an equivalent number of cells were noted in cultures receiving both OGF and taxol (10^-10 M) compared to those receiving the higher concentration of taxol (10^-9 M) alone (Figure 3.1A).

To establish the efficacy of the combination of OGF with cisplatin on the growth of human ovarian cancer cells, SKOV-3 cultures were treated for 120 h with OGF (10^-6 M), cisplatin (0.01 μg/ml or 0.001 μg/ml; dosages selected because preliminary experiments revealed no logarithmic growth at higher concentrations), OGF and cisplatin, or an equivalent volume of sterile water. Relative to sterile water treated controls, cell number was reduced by OGF (25-37%), cisplatin at either concentration (18-52%), or both OGF and cisplatin (at either concentration) (24-60%) (Figure 3.1B). In cultures receiving a combination of OGF and cisplatin (at either concentration), cell number was reduced 24-46% compared to cells exposed to OGF alone, and decreased 15-30% relative to cells exposed to cisplatin (at either concentration) alone (Figure 3.1B). At all time points evaluated, an equivalent number of cells were noted in cultures receiving OGF and cisplatin (0.001 μg/ml) compared to those receiving the higher concentration of cisplatin (0.01 μg/ml) alone (Figure 3.1B).
Opioid Receptor Mediated Effects of OGF, But Not Taxol or Cisplatin

To determine whether the effects of OGF, taxol, or cisplatin, were mediated by opioid receptors, cultures were exposed to the short acting opioid receptor antagonist Nal. Cells were treated for 96 h with OGF (10^{-6} M), Nal (10^{-6} M), taxol (10^{-9} M or 10^{-10} M), cisplatin (0.01 µg/ml or 0.001 µg/ml), OGF and Nal, taxol and Nal, cisplatin and Nal, or an equivalent volume of sterile water. Relative to control levels at 96 h, addition of OGF, taxol, or cisplatin inhibited cell number by 26-49% (Figures 3.1C, D). Addition of Nal, at a concentration that alone had no effect on cell number, completely blocked the growth inhibitory effects of OGF, but had no influence on the inhibitory action of taxol or cisplatin (Figures 3.1C, D).

Reversibility of the Inhibitory Effects of OGF, But Not Taxol or Cisplatin

To establish whether the inhibitory effects of OGF, taxol, or cisplatin on cell number could be reversed by withdrawing cells from drug exposure, cultures of SKOV-3 cells were exposed for 72 h to OGF (10^{-6} M), taxol (10^{-9} M or 10^{-10} M), cisplatin (0.01 µg/ml or 0.001 µg/ml), or an equivalent volume of sterile water. At 72 h, one-half of the groups stopped receiving drug treatment, and media was replaced without compounds (i.e. OGF-reversal, taxol-reversal, cisplatin-reversal); the remaining cultures continued to receive media and compounds. At 96 and 120 h, the OGF-reversal group had 40% and 42% more cells, respectively, than in the group continuing to receive OGF, and at 120 h had a comparable number of cells as sterile water treated cultures (Figures 3.1E-H). However, the taxol and cisplatin reversal groups did not differ from cultures continuing to be treated with taxol or cisplatin, respectively, and remained significantly decreased from sterile water controls (Figures 3.1E-H).

Mechanism of Enhanced Growth Inhibition In Vitro

To evaluate the mechanism(s) by which treatment with both OGF and taxol, or a combination of OGF and cisplatin, decrease SKOV-3 cell number, DNA synthesis and cell survival were evaluated. Compared to the BrdU labeling index of sterile water treated cells (28.5 ± 2.1%), cells treated with OGF (10^{-6} M), taxol (10^{-9} M or 10^{-10} M), or cisplatin (0.01 µg/ml or 0.001 µg/ml) had 31-46% less cells incorporating BrdU (Figures 3.2A, B). Cultures receiving both OGF and taxol (10^{-10} M), or OGF in combination with taxol (10^{-9} M), had 45% and 60%, respectively, fewer cells labeled with BrdU relative to
cells exposed to OGF, and 56% and 60%, respectively, less cells labeled with BrdU relative to cultures treated with taxol alone (Figure 3.2A). Cultures receiving OGF and cisplatin (0.01 µg/ml) had decreased labeling indexes of 47% and 43% relative to cultures receiving either OGF or cisplatin (0.01 µg/ml), respectively. However, comparable BrdU labeling indexes were noted in cultures exposed to OGF and cisplatin (0.001 µg/ml) in contrast to cells receiving OGF or cisplatin (0.001 µg/ml) alone (Figure 3.2B).

Examination of apoptosis or necrosis in SKOV-3 cells treated with OGF (10^{-6} M), taxol (10^{-9} M or 10^{-10} M), cisplatin (0.01 µg/ml or 0.001 µg/ml), OGF and taxol, or OGF and cisplatin, revealed less than 0.1% positive cells for apoptosis and necrosis, and these data were comparable to values obtained with cells subjected to sterile water (data not shown).

**OGF Inhibits Established Ovarian Cancer, and Can Be Combined with Taxol or Cisplatin for an Additive Inhibitory Effect on Tumor Progression**

Beginning 2 days after initiation of treatments and persisting throughout the study, tumor volumes in mice with established s.c. ovarian xenografts were reduced by treatment with OGF (26-50%), taxol (22-50%), cisplatin (31-58%), both OGF and taxol (21-62%), and a combination of OGF and cisplatin (30-70%) compared to control animals receiving saline (Figures 3.3A, B). In mice receiving OGF in combination with taxol, tumor volumes were reduced 11-28% from mice treated with OGF alone beginning on day 16 and continuing through the remainder of the experiment. Moreover, mice treated with OGF and taxol had tumor volumes that were decreased 19-21% from mice receiving taxol alone beginning on day 7 (Figure 3.3A). Mice administered OGF and cisplatin had tumor volumes that were reduced 15-50% from mice treated with OGF alone beginning on day 14, and decreased 19-44% in comparison to mice treated with cisplatin alone beginning on day 7 (Figure 3.3B).

On the day of termination (day 37), mice from all treatment groups displayed a visible reduction in tumor size (Figure 3.3C) compared to controls subjected to saline, with decreases in both tumor volume (28-64%, Figure 3.3D) and tumor weight (32-70%, Figure 3.3E) recorded. Relative to tumor bearing mice treated with either OGF or taxol alone, animals exposed to the combination of OGF and taxol had reductions in tumor
volumes (24% and 29%, respectively) and tumor weights (34% and 28%, respectively) (Figures 3.3D, E). Similarly, relative to mice treated with either OGF or cisplatin, OGF and cisplatin in combination depressed tumor volumes (48% and 44%, respectively) and tumor weights (56% and 46%, respectively) (Figures 3.3D, E).

**Effects of Treatments on Body Weight and Gross Observations**

Although all mice weighed approximately 18 to 20 g at the beginning of the experiment (Figure 3.4), mice receiving cisplatin had a 14-21% reduction in body weight compared to saline administered controls beginning on day 9 and extending through day 16 of the study. Mice receiving the combination of OGF and cisplatin also were reduced from saline treated controls in body weight (11-13%) from days 9 through 14 of the study. However, mice exposed to both OGF and cisplatin weighed 7-12% more than mice treated with cisplatin alone. Body weights of mice administered OGF, taxol, or the combination of OGF and taxol, were comparable to saline controls throughout the study.

Terminal spleen weights were similar in all groups of mice (data not shown), and behavioral abnormalities were not evident. Metastasis or lesions were not noted in mice from any group.

**Mechanism of Enhanced Tumor Growth Inhibition In Vivo**

Examination of apoptosis by TUNEL assay revealed similar levels of programmed cell death in tumors taken from mice treated with either OGF or saline (Figure 3.5A). Mice treated with taxol or cisplatin, either alone or in combination with OGF, had 97-122% and 239-273%, respectively, more apoptotic cells compared to saline administered controls (Figure 3.5A). With respect to cells in tumors undergoing DNA synthesis, a reduction of 34-51% was noted in all treatment groups compared to saline controls (Figure 3.5B). The BrdU labeling index in mice receiving the combination of OGF and taxol, or both OGF and cisplatin, was reduced 26% compared to mice treated with taxol or cisplatin alone (Figure 3.5B). Levels of DNA synthesis in tumors were similar in mice treated with OGF, the combination of OGF and taxol, or the combination of OGF and cisplatin (Figure 3.5B). With respect to the density of blood vessels in tumors, blood vessel density was reduced 55-86% in all treatment groups relative to animals exposed to saline (Figure 3.5C). Blood vessel density was
decreased approximately 43% in mice treated with the combination of OGF and taxol, and decreased 69% in mice treated with the combination of OGF and cisplatin, compared to mice administered individual treatments (Figure 3.5C).

**The Presence and Expression of OGF and OGFr in Xenografts**

To evaluate the distribution and relative expression of OGF in xenografts, semiquantitative immunohistochemistry was performed. OGF was visible in the cytoplasm and a speckling of immunoreactivity often was noted in cell nuclei (Figure 3.6A). Tumors processed with only secondary antibody showed no staining (Figure 3.6A inset). OGF distribution and immunofluorescence (mean gray value) did not differ between any group (Figure 3.6B).

To examine OGFr distribution and relative expression, immunohistochemistry, Western blotting, and receptor binding assays were performed on xenografts. The cellular location of OGFr was similar in all groups, with immunoreactivity detected in the cytoplasm and nucleus (Figure 3.6C). Tumors processed with only the secondary antibody showed no staining (Figure 3.6C inset). Relative to saline administered controls, OGFr expression in mice treated with OGF, both OGF and taxol, or OGF combined with cisplatin was decreased 47-51% using semiquantitative immunohistochemistry (Figure 3.6D). Further evaluation of OGFr expression using Western blotting, showed that mice treated with OGF had an 81% reduction in OGFr expression in their tumors compared to saline administered controls (Figures 3.6E, F).

Receptor binding assays indicated specific and saturable binding for OGFr in tumors of all groups, with a one site model of binding recorded (Figure 3.6G). Binding capacity (B$_{\text{max}}$) values were markedly reduced (44-51%) in mice treated with OGF, both OGF and taxol or OGF in combination with cisplatin, compared to control animals receiving saline (Figure 3.6G). However, binding affinity (K$_{d}$) for OGFr did not differ among treatment groups and ranged from 2.0 nM to 7.5 nM (data not shown).
Discussion

The present study demonstrates for the first time that OGF biotherapy, alone or in combination with taxol or cisplatin chemotherapies, has a potent inhibitory effect on the proliferation of a human ovarian cancer cell line, SKOV-3. The repressive effects of OGF, taxol, or cisplatin alone on cell number were consonant with previous reports with respect to a variety ovarian cancer cells in vitro [11, 35, 36]. We now have discovered that the anti-proliferative effect of the combination of OGF with taxol or cisplatin was greater than that of the individual drugs, indicating an additive action of these biotherapeutic and chemotherapeutic agents. Indeed, cell number was comparable between cultures receiving taxol or cisplatin alone and cultures receiving OGF in combination with a 10-fold lower concentration of these agents. Two major differences in the effects of OGF on cell growth were noted in contrast to taxol or cisplatin. First, the inhibition of cell number by OGF, but not taxol or cisplatin, was mediated by opioid receptors, with the opioid antagonist Nal neutralizing the repercussions of OGF in the absence of a growth effect of Nal alone. Second, the suppressive effect of OGF on cell proliferation was reversed when media was replaced without drug, and cell number returned to normal. In contrast, a discontinuation of taxol or cisplatin treatment did not change the growth characteristics of SKOV-3 cells, with these cultures resembling those that continued to receive taxol or cisplatin. Moreover, the number of ovarian cancer cells in cultures undergoing reversal from OGF was significantly greater than in the taxol or cisplatin reversal groups. These results show that the combination of two treatment modalities with differing mechanisms, biotherapy and chemotherapy, act in a synergistic fashion to impede the growth of human ovarian cancer cells in tissue culture.

The results of this study make the seminal observation that daily exposure to OGF markedly impedes the progression of human ovarian tumorigenesis. This was evident from our data demonstrating that even 5 weeks after initiating peptide treatment both tumor volume and tumor weight were reduced one-third from tumor-bearing mice injected with saline. Moreover, the magnitude of effects of OGF on tumorigenicity was similar to that of standard of care agents: taxol and cisplatin. We also discovered that the combination of OGF with either taxol or cisplatin had greater anti-tumor activity than individual agents alone. For example, the additive inhibitory effects of OGF and
cisplatin on tumor weight were almost 2-fold greater compared to either agent alone. Tolerance in suppressing tumor growth, at least within the 5-week period of our observation, was not observed in any of the treatment groups. These results with a xenograft model in mice support and extend our findings from the tissue culture model. Additionally, our data validate not only that OGF has a potent antitumor effect on ovarian carcinogenesis, but that a combination of biotherapy with OGF and chemotherapy with taxol or cisplatin, have a cooperative effect in retarding the growth of this lethal disease.

Although both OGF and taxol in the concentrations and regimens used in this study were not overtly toxic to mice with xenografts of ovarian cancer, animals subjected to cisplatin had a notable reduction in body weight. This systemic toxicity from cisplatin was diminished by simultaneous administration of OGF, indicating that this opioid peptide has the capacity to protect against toxicological insults. The amelioration of cisplatin toxicity by OGF, however, was not accompanied by a diminution of the antitumor action of cisplatin. In fact, the combination of OGF and cisplatin had an effect on tumor growth (i.e. weight, volume) that exceeded cisplatin or OGF alone. The mechanism of protection afforded by OGF against cisplatin toxicity is unknown. However, the alleviation of toxicity of one agent by the administration of another drug is not without precedence [37], and in fact has been observed when OGF was administered in combination with a toxic regimen of taxol in mice with xenografts of squamous cell carcinoma of the head and neck [38]. The finding of protection afforded by OGF from the side effects of cisplatin may allow higher doses of cisplatin to be administered to improve the therapeutic efficacy of this agent. This may be advantageous, as the success of chemotherapy is often limited by an intrinsic resistance of cancer cells [39], and the possibility of increasing the concentration of drugs without an accompanying increase in cytotoxicity would be advantageous.

In the present study, the mechanism for enhanced growth inhibition of SKOV-3 cells in tissue culture by a combination of OGF with taxol or cisplatin was not associated with induction of apoptosis or necrosis, at least at the low dosages of taxol and cisplatin used herein, but instead was related to an additive inhibitory effect on DNA synthesis. In vivo, the mechanism for enhanced inhibition of tumorigenesis by OGF in combination
with taxol, or both OGF and cisplatin, appears to be related to a number of mechanisms. First, OGF and/or taxol/cisplatin reduced DNA synthesis and angiogenesis in tumors. Second, taxol and cisplatin induced apoptosis. These effects of OGF, taxol, and cisplatin are consistent with previous observations. Taxol is a chemotherapeutic agent that stabilizes microtubules, thereby preventing microtubule depolymerization and chromosome segregation. These actions lead to an arresting of the cell cycle in the G2/M phase that ultimately results in cell death [40]. Cisplatin also arrests DNA replication in a cell-phase specific manner at G2/M, but induces apoptosis by binding to DNA and nuclear proteins to form DNA intra and interstrand crosslink [41, 42]. OGF, on the other hand, stalls cells at the G1/S phase of the cell cycle through upregulation of p16/p21 CKI pathways, delaying cell proliferative processes (e.g., DNA synthesis, angiogenesis) without influencing cell survival [11-15, 43, 44]. Thus, the individual effects of biotherapy and chemotherapy are enhanced by combining agents that target similar and differing fundamental biological processes.

Another important finding of the present study is that OGF treatment administered either alone or in combination with taxol or cisplatin reduces the expression and binding of OGFr. Despite this reduction in OGFr, the OGF-OGFr axis remains functional, as demonstrated by the continued inhibition of tumor volume and weight seen with OGF treatment in animals with xenografts. This finding of a reduction in OGFr with OGF treatment is consonant with a previous report [27], wherein OGFr binding was reduced in pancreatic tumors removed from xenografted mice that were treated chronically with OGF. Furthermore, treatment with other opioids has been documented to reduce the expression of classic opioid receptors [45, 46]. Therefore, in the present study it can be postulated that OGFr expression adjusts to the excess of OGF by administration of exogenous peptide, resulting in a downregulation of OGFr.

In previous studies investigating the effects of OGF on carcinogenesis, the paradigm used was to initiate administration of the peptide concomitant with tumor cell inoculation. These reports concluded that OGF could suppress tumor appearance and delay tumor progression of a wide variety of cancers when utilized in this manner [25-27]. In contrast to earlier investigations, treatment with OGF alone or in combination with taxol or cisplatin, in the present study commenced when tumors were measurable.
These results reveal that OGF not only can influence early tumorigenic events, but can exert a potent action on an established cancer. With these observations in mind, it may be conjectured that OGF can be used as an antitumor agent in ovarian cancer prior to tumor expression as a prophylactic therapy. Moreover, our present findings would suggest that patients with established disease or following cytoreductive surgery could benefit from OGF biotherapy alone or in combination with standard of care drugs.

This report showing that OGF alone and in combination with two chemotherapeutic agents has a marked antitumor effect with regard to human ovarian cancer has several clinical implications. OGF has been documented to be non-toxic in Phase I trials, both by the infusion and s.c. routes of administration [47]. OGF also has been reported to have efficacy in extending survival of patients with advanced pancreatic cancer in a Phase II clinical trial [48]. These previous reports documenting that OGF is non-toxic and efficacious, make the transition from the laboratory to the clinic in terms of using OGF alone or combined with standard of care drugs more feasible for the treatment of ovarian cancer. Our results suggest that OGF could be used under three different circumstances: i) as a prophylactic agent, particularly in patients with a family history of ovarian cancer, ii) as a first line treatment, alone or in combination with standard of care drugs, following cytoreductive surgery, and iii) following relapse when all other treatments are palliative.
Figures and Legends

**Figure 3.1.** Effects of OGF in combination with taxol or cisplatin on the growth of SKOV-3 human ovarian cancer cells. Cells were treated with OGF (10^{-6} M), taxol (10^{-9} M or 10^{-10} M), cisplatin (0.01 μg/ml or 0.001 μg/ml), OGF and taxol, OGF and cisplatin, or an equivalent volume of sterile water (Co). Media and compounds were replaced daily unless otherwise indicated. (A, B) Growth of cells subjected to OGF alone or in combination with taxol (A) or cisplatin (B) over a 120 h period. (C, D) Opioid receptor mediation of the growth inhibitory effects of either OGF, taxol, or cisplatin. Cell number following 96 h of treatment with OGF, taxol, cisplatin, the opioid antagonist Nal (10^{-6} M), or the combination of Nal with these compounds. (E, F) Growth of SKOV-3 cells in reversibility experiments with OGF, taxol, or cisplatin for 72 h; at 72 h, a subset of cultures continued to receive treatments for an additional 48 h, while the other cultures were administered sterile water for 48 h (Reversal). (G, H) Cell number at 120 h in cultures from the reversibility experiments. Values represent mean ± SE cell counts for at least 2 aliquots/well and 2 wells/treatment/timepoint. Significantly different from Co at *p<0.05, **p<0.01, and ***p<0.001, from OGF at ^p<0.05, ^^p<0.01, and ^^^p<0.001, and from taxol or cisplatin at +p<0.05, ++p<0.01, and +++p<0.001. NS = not significant.
Figure 3.2. Mechanism of enhanced growth inhibition \textit{in vitro} by both OGF and taxol, or a combination of OGF and cisplatin. (A, B) Evaluation of DNA synthesis (% BrdU incorporation) in SKOV-3 cells treated with OGF, taxol, cisplatin, OGF and taxol, OGF and cisplatin, or an equivalent volume of sterile water (Co) for 120 h. Media and compounds were replaced daily. Cells were pulsed with BrdU (30 μM) for 3 h prior to fixation and processed for BrdU immunoreactivity. Values represent %BrdU incorporation (means ± SE) determined for at least 500 cells on 10 fields/coverglass and 2 coverglasses/treatment group. Significantly different from Co at ***$p<0.001$, from OGF at ^^$p<0.01$ and ^^^$p<0.001$, and from taxol or cisplatin at +$p<0.01$ and +++$p<0.001$. 

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Figure 3.3. Growth of subcutaneous xenografts with SKOV-3 cells in mice treated with OGF, taxol, cisplatin, or OGF in combination with taxol or cisplatin chemotherapy. When tumors became visible (day 0), animals were injected with either OGF (10 mg/kg, daily), taxol (3 mg/kg, days 0, 7, 14, 21, 28, 35), cisplatin (4 mg/kg, days 0, 7), taxol and OGF, cisplatin and OGF, or an equivalent volume of saline (daily). (A, B) Tumor volumes were assessed 3x/week. Data for the saline and OGF groups in A and B are from the same mice. (C) Representative images of tumors before and after removal from mice following 37 days of treatment. (D) Terminal tumor volume (mm$^3$). (E) Terminal tumor weight (g). Values represent means ± SE for 12 mice/group. Significantly different from saline at *p<0.05, **p<0.01, and ***p<0.001, from OGF at ^p<0.05, ^^p<0.01, and ^^^p<0.001, and from taxol or cisplatin at +p<0.05 and ++p<0.01.
Figure 3.4. Body weight of mice with subcutaneous xenografts of SKOV-3 cells treated with OGF, taxol, cisplatin, or OGF in combination with taxol or cisplatin, commencing when tumors were visible. Values represent means ± SE body weight for 12 mice/group. Significantly different from saline at **p<0.01 and ***p<0.001, and from cisplatin at +p<0.05 and ++p<0.01.
Figure 3.5. Mechanism of tumor growth inhibition by treatment with OGF, taxol, cisplatin, both OGF and taxol, or OGF in combination with cisplatin: effects on apoptosis, DNA synthesis, and angiogenesis. Treatments were initiated when tumors were visible (day 0) and tumor tissue was assessed 37 days later. (A) Number of apoptotic cells per 0.003 mm$^2$, as measured by TUNEL assay. (B) % BrdU labeling. (C) Number of blood vessels per 0.16 mm$^2$, as assessed by Hematoxylin/Eosin staining to identify endothelial cell-lined blood vessels. Values represent means ± SE determined from at least 10 random fields from the periphery of 2 tumor sections/mouse and 2 mice/group. Significantly different from saline at ***p<0.001, from OGF at ^^-p<0.01 or ^^-^-p<0.001, and from taxol or cisplatin by +p<0.05 or ++p<0.01.
Figure 3.6. The distribution and expression of OGF and OGFr in xenografts of SKOV-3 cells. Mice were treated with OGF, taxol, cisplatin, or OGF in combination with taxol or cisplatin when tumors were visible. (A, C) Photomicrographs taken at the same exposure time of tumors on the day of sacrifice (day 37) stained with antibodies (1:200) to OGF (A) or OGFr (C). Rhodamine conjugated IgG (1:1000) served as the secondary antibody and nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (insets). Bar = 10 μm. (B, D) Semiquantitative measurement of OGF (B) and OGFr (D) staining intensity (mean gray value) from at least 10 fields from 2 sections/tumor and 3 mice/group. (E, F) Western blot of the 62 kDa band of OGFr (E) and densitometric analysis (F) normalized to β-actin from 2 independent experiments. (G) Saturation isotherms calculating the binding capacity (B_max) of OGFr in xenografts from at least 3 independent assays performed in duplicate. Data represent means ± SE. Significantly different from saline at **p<0.01 and ***p<0.001.
References


[20] F. Cheng, P.J. McLaughlin and I.S. Zagon, “Regulation of cell proliferation by the opioid growth factor is dependent on karyopherin beta and Ran for


Chapter 4: LDN In Vitro

Rationale
Upregulation of the OGF-OGFr through treatment with OGF, inhibits human ovarian cancer cell proliferation and tumor growth. To evaluate another potential way by which the OGF-OGFr axis can be pharmacologically modulated and serve as a novel therapeutic option for the treatment of ovarian cancer, low dose naltrexone (LDN), which is known to block opioids and opioid receptors for a short period of time (4-6 h/day), was studied. During this period of blockade, there is an expected compensatory upregulation in the production of endogenous opioids and opioid receptors (including OGF and OGFr). When LDN is no longer present, there is thought to be a robust interaction between OGF and OGFr that results in a marked depression of cell proliferation (10-20 h/day). The present study evaluated whether LDN inhibits human ovarian cancer cell proliferation and established the mechanism of action using a tissue culture model of LDN where cultures are treated for a short period of time with NTX to mimic LDN’s action in vivo.
Low dose naltrexone (LDN) targets the OGF-OGFr pathway to inhibit cell proliferation: Mechanistic evidence from a tissue culture model

Submitted for publication

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Abstract
Naltrexone (NTX) is an opioid antagonist that inhibits or accelerates cell proliferation in vivo when utilized in a low (LDN) or high (HDN) dose, respectively. The mechanism of this opioid antagonist on growth is not well understood. We established a tissue culture model of LDN and HDN using short term and continuous opioid receptor blockade, respectively, with NTX in human ovarian cancer cells, and found that the duration of opioid receptor blockade determines cell proliferative response. The alteration of growth by NTX was ubiquitous. The opioid growth factor (OGF; [Met\(^5\)]-enkephalin) and its receptor, OGFr, were responsible for mediating the action of NTX on cell proliferation. NTX upregulated OGF and OGFr at the translational but not transcriptional level. The mechanism of inhibition by short term NTX required p16 and/or p21 cyclin-dependent inhibitory kinases, but was not dependent on cell survival (necrosis, apoptosis). Sequential administration of short term NTX and OGF had a greater inhibitory effect on cell proliferation than either agent alone. Given the parallels between short term NTX in vitro and LDN in vivo, we now demonstrate at the molecular level that the OGF-OGFr axis is a common pathway that is essential for the regulation of cell proliferation by NTX.
Introduction

Naltrexone (NTX) is a general opioid receptor antagonist that is devoid of intrinsic activity, and blocks opioids from opioid receptors.\textsuperscript{1-4} Opioid antagonist modulation of endogenous opioid systems has been used to decipher the function of opioid peptide-opioid receptor interactions in a number of biological processes and diseases.\textsuperscript{5-15} One function of endogenous opioids is the regulation of growth through a tonically active inhibitory pathway.\textsuperscript{16} Systemic exposure to a high dose of NTX (HDN) or a low dose of NTX (LDN) given multiple times each day, continuously blocks opioid receptors and accelerates cell proliferation and growth.\textsuperscript{13, 15, 17-21} In contrast, intermittent or short term opioid receptor blockade, achieved by daily administration of LDN or a low dose of naloxone, blocks opioid peptide-opioid receptor interactions for a short duration each day (e.g., 4 to 6 hours), and inhibits cell proliferation and growth in the interval when the opioid antagonist is no longer present.\textsuperscript{6, 15, 18, 22-24}

The opposing effects on growth observed with continuous and intermittent opioid receptor blockade are related to the pharmacological action of opioid antagonists. The response to opioid antagonist administration is a compensatory upregulation in the production of opioid peptides and opioid receptors.\textsuperscript{20, 25-27} Unlike continuous opioid receptor blockade, wherein the upregulated opioid peptides and receptors do not have the opportunity to interface, pharmacokinetic,\textsuperscript{28} as well as nociceptive and functional,\textsuperscript{15, 18} studies have shown that blockade of opioids from opioid receptors for a short period of time (4 to 6 hours) each day provides an 18 to 20 hour window where the elevated opioids and opioid receptors can interact to elicit an exaggerated response (e.g. depression in cell proliferation).\textsuperscript{23, 29-31} In the case of neoplasia, for example, persistent blockade of opioid receptors from endogenous opioids has a profound effect on oncogenesis by accelerating tumor appearance and growth.\textsuperscript{13, 15, 18, 22} However, a temporary blockade of opioid receptors from native opioids markedly suppresses the onset and progression of carcinogenesis.\textsuperscript{6, 13, 15, 18, 22, 23}

One particular endogenous opioid-opioid receptor system that serves as a determinant of cell proliferation and growth, and is modulated by NTX, is the opioid growth factor (OGF) and its receptor, OGFr.\textsuperscript{19, 21} OGF, chemically termed \([\text{Met}^5]-\text{enkephalin}\), is a constitutively expressed native opioid peptide that is autocrine
OGF interacts with OGFr (a non-classical opioid receptor) to delay the G1/S phase of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways, and inhibits cell proliferation in normal and a variety of neoplastic cells. An increase in OGF-OGFr activity in cancer cells by the addition of exogenous OGF, treatment with imidazoquinoline compounds such as imiquimod and resiquimod, or transfection of sense cDNA for OGFr, depresses cell proliferation. In contrast, attenuation of the OGF-OGFr axis in cancer cells through disruption of peptide-receptor interfacing by continuous exposure to NTX, neutralization of OGF by antibodies to this peptide, or a decrease in OGFr by antisense cDNA or siRNA stimulates cell proliferation.

A number of lines of evidence suggest that NTX regulates growth through modulation of opioid peptide and opioid receptor interactions, and specifically through the OGF-OGFr axis. First, the effects of opioid antagonists on growth are stereospecific, indicating that the action of these agents is dependent on an opioid receptor. Second, the duration of opioid receptor blockade, rather than drug dosage, is a determinant of the direction of growth effects, implying that these antagonists have an indirect action. Third, OGF is the only opioid peptide that has been found to suppress cell proliferation. Fourth, the influence of OGF is mediated by an opioid receptor. Fifth, knockdown of OGFr blocks the effects of OGF, indicating that this opioid receptor is responsible for mediating peptide action. Sixth, OGF and OGFr have been shown to be upregulated by treatment with NTX. Although these observations provide a compelling argument that the OGF-OGFr axis is fundamental to NTX action with respect to growth, it may be argued that they are circumstantial rather than causal. To determine the mechanism of NTX's effects on growth, we have developed a tissue culture model of this opioid antagonist that parallels in vivo events with respect to LDN by exposing cells for 6 hours to NTX (i.e., short term NTX) and examining the repercussions on cell proliferation. Such a tissue culture model removes the confounding influences introduced by systemic biology, and allows direct observation of mechanistic pathways related to LDN. We now show at a molecular level that LDN specifically targets the OGF-OGFr axis to regulate cell proliferation, and that opioid-based modulation of growth requires both OGF and OGFr.
Materials and methods

Cell culture
Human cancer cell lines SKOV-3, OVCAR-3, MiaPaCa-2, and HCT-116 were obtained from the American Type Culture Collection (Manassas, VA), while SCC-1 was provided by Dr. T. Carey (Director of the University of Michigan Cancer Research Laboratory). Cells were grown in a humidified atmosphere of 5% CO$_2$/95% air at 37°C in the following media: Dulbecco’s medium (MiaPaCa-2, SCC-1), RPMI 1640 medium (OVCAR-3, SKOV-3), and McCoy’s 5a medium (HCT-116). All media was supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 units/mL penicillin, 5 μg/mL streptomycin, and 10 mg/mL neomycin) unless otherwise noted.

Cell growth
Log phase cells were plated and counted 24 hours later (time 0) to determine seeding efficiency. Cultures were treated with NTX (10$^{-5}$ M) or an equivalent volume of sterile water. At the end of 6 hours, the media containing compound was removed and replaced with media either lacking NTX (short term NTX) or containing NTX (continuous NTX). Media and compounds (when applicable) were replaced daily. All compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. At designated times, cells were harvested with trypsin, stained with trypan blue to evaluate cell viability, and counted with a hemacytometer. At least 2 aliquots per well and 2 or more wells/treatment/timepoint were sampled.

Antibody neutralization
Endogenous OGF was neutralized with a polyclonal antibody to this peptide (CO172, 1:200); pre-immune rabbit serum (IgG, 1:200) and sterile water treated cells served as controls. Antibody, IgG, and media were changed daily, and cells counted after 72 hours.

siRNA knockdown
Cells were transfected for 24 hours with 20 nM concentrations of one of the following siRNAs: MOR, DOR, or KOR (Santa Cruz Biotechnology, Santa Cruz, CA), OGFr (Ambion, Austin TX), p16 or p21 (Santa Cruz Biotechnology), using Oligofectamine...
reagent (Invitrogen, Carlsbad, CA). Cells were collected for growth curves or Western blotting to determine the level of protein knockdown 72 hours after the start of transfection. Two independent experiments were conducted.

Protein isolation and Western blotting
The level of protein knockdown resulting from siRNA transfection with MOR, DOR, KOR, OGFr, p16 or p21, as well as expression of OGFr following treatment with NTX, was determined by Western blotting following published procedures. In brief, cells were solubilized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN). Protein (60 μg) was subjected to 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies (1:200) against OGFr, MOR, DOR, KOR, or p16 (Santa Cruz Biotechnology), or p21 (BD PharMingen, San Diego, CA). Optical densities were normalized to β-actin (1:5000, Sigma-Aldrich, St. Louis, MO), and the percent change in expression was calculated by dividing the normalized values of experimental samples to that of controls. Means and SE were determined from 2 independent experiments.

RNA isolation, Northern blotting, and reverse transcriptase-polymerase chain reaction (RT-PCR)
To evaluate OGFr mRNA levels, Northern blotting was performed according to Zagon et al. Total RNA was extracted using the Paris Kit (Ambion), separated on an agarose gel, transferred to a nylon membrane (Immobilon, Bio-Rad Laboratories, Hercules, CA), and probed with 32P-dCTP-OGFr cDNA or 32P-dCTP- GAPDH cDNA. Optical densities were normalized to GAPDH and the percent change in expression was calculated by dividing the normalized values of experimental samples to that of sterile water treated controls. Means and SE were ascertained from at least two independent experiments.

To determine PPE mRNA expression levels, RT-PCR was performed. cDNA was synthesized from 0.7 μg denatured total cellular RNA and reverse transcribed in a final volume of 20 μl using the superscript III kit (Invitrogen). The cDNA equivalent of 0.2 μg total RNA was amplified by PCR in a final volume of 50 μl buffer containing 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 2 mM MgCl2, dNTP’s (0.25 mM each), 1 μM of each primer, and 0.25 U Taq Polymerase. PCR was carried out for 35 cycles in a Perkin Elmer thermocycler (Perkin Elmer, Foster City, CA). The first cycle consisted of
denaturation at 94°C for 5 min, annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. For the next cycles, the denaturation time was 1 min, and during the last cycle, primer extension lasted 10 min. As a control, β-globulin was amplified under the same conditions as described for PPE. The 21-mer sense oligonucleotide GCGACGGTGAGGCCCTACGTC and 23 mer antisense oligonucleotide AGCCGGGTTCCAGACACGACTCTA were used to amplify a 113 bp PPE fragment, while the 20-mer sense oligonucleotide ACACAACGTGTCTACAGC and 20-mer antisense CAACTTCATCCACGTTCACC were used to amplify a 100 bp β-globulin fragment. PCR products were run on a 2% agarose gel, visualized with ethidium bromide, and the optical density of each band was determined and analyzed by QuickOne (Bio-Rad Laboratories, Hercules, CA). Each value was normalized to β-globulin. At least two samples were evaluated for each group, and means and SE were determined from three independent experiments.

Semiquantitative immunohistochemistry
To examine the distribution and relative levels of OGF and OGFr, log-phase cells grown on 22 mm round coverglasses were fixed and stained with antibodies to OGF and OGFr according to published procedures. Polyclonal antibodies to OGF and OGFr were generated in the laboratory and have been fully characterized. Controls included cells incubated only with secondary antibodies. At least 3 coverglasses were examined. 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 3 coverglasses/group.

Radioimmunoassay
To determine the levels of OGF secreted from cells into culture media, log phase SKOV-3 cells were treated for 6 hours with NTX or an equivalent volume of sterile water in serum-free media. At 6 hours, NTX containing media was removed and replaced with serum-free media either lacking or containing NTX as previously described. Media was not replaced from this time point onward in the experiment so as to measure accumulated levels of secreted OGF. At designated times, 1 mL samples of media were collected and assayed for OGF using a radioimmunoassay kit from Peninsula Laboratories (San Carlos, CA). Sterile serum free media was also monitored as a
control. At least two samples were evaluated for each group, and each sample was assayed in duplicate.

**OGFr binding assays**

Receptor binding assays for OGFr were performed in log phase cells treated with NTX (10^{-5} M) for either a short term or continuously using custom synthesized $[^3H]^{-}\text{[Met}^5\text{-enkephalin}}$ (Perkin Elmer, Waltham, MA; 52.7 Ci/mmol). Non-specific binding was measured in the presence of unlabeled [Met$^5$]-enkephalin. Saturation binding isotherms were generated using GraphPad Prism software (La Jolla, CA), and independent assays were performed in duplicate at least 3 times.

**DNA synthesis, apoptosis, and necrosis**

To evaluate DNA synthesis, cells were seeded on 22 mm diameter coverglasses in 6-well plates and, at designated times, pulsed with 30 μM BrdU (Sigma-Aldrich) for 3 hours. Preparations were fixed with 10% neutral buffered formalin and stained with antibodies to BrdU (Invitrogen). Using similar preparations, apoptosis was assessed using TUNEL (Trevigen, Gaithersburg, MD) according to the manufacturer’s instruction. Necrosis was ascertained by trypan blue exclusion staining.

**Chemicals**

The following compounds were obtained from the indicated sources: [Met$^5$]-enkephalin (OGF), [Leu$^5$]-enkephalin (leu enk), [D-Pen$^{2,5}$]-enkephalin (DPDPE), [D-Ala$^2$,MePhe$^4$,Glyol$^5$]-enkephalin (DAMGO), β endorphin (β-endo), NTX, naloxone, dynorphin A1-8 (dynorphin), morphine sulfate (morphine), endomorphin 1 (endo-1), endomorphin 2 (endo-2), Sigma-Aldrich; U69,583, Upjohn Diagnostics (Kalamazoo, MI).

**Statistical analysis**

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

Short term NTX treatment depresses the growth of cancer cells

To determine whether the duration of NTX treatment affects the growth of cancer cells, SKOV-3 cultures were treated with $10^{-5}$ M NTX, a concentration that is not toxic but has marked efficacy on growth, either i) once for 6 hours (short term NTX), ii) 6 hours every 24 or 48 hours, or iii) continuously for 24 hours on a daily basis (i.e., continuous NTX). A single application of short term NTX, as well as exposure to short term NTX every 48 hours, inhibited cell number by 22 to 29% from control levels at 48 to 96 hours (Figure 4.1A). In contrast, cultures treated with short term NTX every 24 hours had a comparable number of cells as controls at all time points evaluated. Continuous exposure to NTX increased cell number by 22 to 42% from control levels at 48 to 96 hours.

To ascertain how long short term NTX suppresses the proliferation of SKOV-3 cells, cultures were exposed only once for 6 hours with NTX or sterile water and monitored daily for cell number from 96 to 192 hours. Cell number was reduced from control levels at 96 and 120 hours. However, from 144 hours onward, cell number in short term NTX treated cultures was comparable to that of controls (data not shown).

In a subsequent set of experiments, the growth effects of NTX given daily for a period of time shorter than 6 hours were assessed. Cultures treated daily for 1, 2, or 3 hours and examined at 72 hours were decreased by 24 to 33% from controls subjected to sterile water (Figure 4.1B). Cultures treated with NTX on a daily regimen for 4, 5, or 6 hours had comparable cell numbers to control levels.

Short term opioid receptor antagonism with naloxone inhibits cancer growth

To determine whether another opioid antagonist administered for a short term alters growth, SKOV-3 cultures were treated with a single application of naloxone for only 6 hours. Administration of $10^{-3}$ M and $10^{-4}$ M naloxone inhibited cell number by 28 and 25%, respectively, at 72 hours (Figure 4.1C). Cultures subjected to $10^{-5}$ M or $10^{-6}$ M naloxone, however, displayed no differences in cell number from controls receiving an equivalent volume of sterile water.
OGF is the endogenous opioid peptide specific for growth inhibition of cancer cells

To determine which opioid(s) is(are) responsible for the growth inhibition recorded with short term NTX treatment, SKOV-3 cultures were exposed continuously for 72 hours to $10^{-6}$ M concentrations of natural or synthetic opioid-related compounds, some specific for $\mu$, $\delta$, and $\kappa$ opioid receptors. OGF was the only opioid that had an effect on cell growth, depressing cell number by 32% compared to controls exposed to sterile water, a level equivalent to that recorded for short term NTX (Figure 4.2A).

To test the specificity of short term NTX on OGF with regard to cell proliferation, an antibody neutralization experiment was performed. At 72 hours, in contrast to a reduction of 27% in cell number for cultures exposed to short term NTX and treated with sterile water or IgG, cells treated with both short term NTX and the antibody to OGF no longer exhibited a reduction in cell number (Figure 4.2B).

Silencing of OGFr, but not classical opioid receptors, blocks the inhibitory action of short term NTX

The requirement of classical and/or non-classical opioid receptors for the ability of short term NTX to inhibit cell proliferation was evaluated at the molecular level using siRNA technology. Western blot analysis revealed that MOR, DOR, KOR, or OGFr siRNA-transfected SKOV-3 cultures had reductions of 64 to 92% in these receptor protein levels relative to untransfected or scrambled siRNA transfected cultures (Figures 4.3A-D). Relative to untransfected or scrambled siRNA cultures, cells transfected with MOR, DOR, or KOR siRNAs had an equivalent number of cells; however, cultures transfected with OGFr siRNA had 42% more cells (Figure 4.3E). The addition of short term NTX inhibited cell number in cultures transfected with scrambled, MOR, DOR, or KOR siRNA by 33 to 42% in comparison to cultures transfected with these same siRNAs and treated with sterile water. However, cell proliferation was not reduced by short term NTX in cultures transfected with OGFr siRNA and, in fact, was 43% greater than untransfected cells exposed to sterile water. For comparative purposes, OGF depressed cell number in untransfected cells, as well as in cultures transfected with MOR, DOR, KOR, or scrambled siRNAs, but not in preparations transfected with OGFr siRNA wherein cell number was 45% greater than in untransfected vehicle treated cultures.
Repercussions of NTX treatment on transcription and translation of OGF

Studies on the expression of OGF and the gene that encodes this peptide, PPE, were evaluated in SKOV-3 cultures administered short term NTX, continuous NTX, or an equivalent volume of sterile water. PPE mRNA expression levels in cells were comparable regardless of treatment with NTX or sterile water at all time points examined (Figure 4.4A). Cellular levels of OGF monitored by semiquantitative immunohistochemistry, however, were increased in both the short term and continuous NTX cultures by 11 to 18% at 24 hours and 22 to 32% at 72 hours from sterile water treated controls (Figure 4.4B). For all cultures, OGF was visible in the cytoplasm, and a speckling of immunoreactivity noted in cell nuclei. Cells processed only with secondary antibody showed no staining.

To examine the effects of NTX treatment on the secretion of OGF from SKOV-3 cells, cultures were treated with this opioid antagonist for either a short or continuous duration; media was changed only at 6 hours. OGF levels in the media were similar in control, short term, and continuous NTX cultures at 6 hours (Figure 4.4C). However, by 24, 48, and 72 hours OGF levels were increased from control values by 23, 25, and 48%, respectively, in media from cultures treated with short term NTX. In media sampled from cultures subjected to continuous NTX, OGF levels were increased 19% at 24 hours, decreased at 48 hours by 32%, and similar to control levels at 72 hours.

Repercussions of NTX treatment on transcription and translation of OGFr

OGFr mRNA levels were evaluated in SKOV-3 cultures administered short term NTX, continuous NTX, or an equivalent volume of sterile water. Expression of OGFr mRNA was comparable in cells treated with either regimen of NTX or sterile water (Figure 4.5A).

Cellular levels of OGFr as detected by semiquantitative immunohistochemistry were increased from control levels in both the short term and continuous NTX cultures at 48 and 72 hours by 13 to 28% (Figure 4.5B). For all cultures OGFr was observed in the cytoplasm, with light speckled staining noted in cell nuclei. Cells processed only with secondary antibody showed no staining. Western blot analysis revealed that cultures treated with short term or continuous NTX were similar in OGFr levels at 6 and
24 hours to that of cultures receiving sterile water, but increased 1.7- to 3.8-fold at 48 and 72 hours (Figure 4.5C).

To further characterize OGFr, receptor binding analysis using nuclear fractions of cells was performed. Binding affinities did not differ between cultures treated with short term NTX, continuous NTX, or sterile water at 72 hours, with $K_d$ values ranging from 4.9 to 5.4 nM (Figure 4.5D). Values for binding capacity of NTX treated cells, however, were increased at 72 hours compared to cultures receiving sterile water ($5.8 \pm 0.8$), with increases of 109% for short term NTX and 46% for continuous NTX recorded.

**The effects of short term NTX on cell proliferation are ubiquitous**

To examine the ubiquity of short term NTX on cell proliferation, human cancer cell lines representing ovarian cancer (OVCAR-3), pancreatic cancer (Mia PaCa-2), squamous cell carcinoma of the head and neck (SCC-1), and colorectal cancer (HCT-116) were treated with short term NTX and examined at 72 hours. Cancer cell lines receiving this regimen of NTX were reduced in cell number by 24 to 31% from their sterile water treated controls, a result similar to that of cultures subjected to OGF (Figure 4.6). In contrast, continuous exposure of these cell lines to NTX increased cell number by 16 to 27% from sterile water controls.

**The combination of short term NTX and OGF inhibits cancer cell number to a greater extent than either agent alone**

To ask the question of whether exogenous OGF enhances the inhibitory effect of short term NTX, OGF was added to SKOV-3 and OVCAR-3 cells 6 hours following a short term exposure to NTX at the time when this opioid antagonist was removed from cultures; OGF and media were replaced daily. Administration of either OGF or short term NTX reduced the number of ovarian cancer cells by 20 to 33% from control levels at 72 hours (Figure 4.7). However, the sequential treatment with short term NTX and OGF depressed the number of SKOV-3 and OVCAR-3 cells by 35 and 61%, respectively, from sterile water treated controls. In contrast to SKOV-3 cells treated with either NTX or OGF, the combination of these agents reduced cell number by 13 to 19%. With regard to OVCAR-3 cells, exposure to both NTX and OGF decreased cell number by 41 to 43% relative to cells treated with just one compound.
Short term NTX alters DNA synthesis but not apoptosis or necrosis

To evaluate the mechanism by which short term NTX inhibits human cancer cell growth, as well as to determine the duration of opioid receptor blockade, DNA synthesis (as measured by BrdU incorporation) in SKOV-3 cultures exposed at 0 hours to short term NTX was monitored. The proportion of BrdU labeled cells in cultures receiving short term NTX was increased by 50, 49, and 18% at 6, 9, and 12 hours, respectively, relative to control levels, whereas at 15 hours, the proportion of BrdU positive cells was comparable to that of the control group (Figure 4.8A). From 18 to 72 hours, the proportion of BrdU labeled cells in cultures that were given short term NTX was decreased by 34 to 48% relative to control values.

To compare the effects of short term NTX, continuous NTX, and OGF treatment on DNA synthesis, SKOV-3 cultures were subjected to short term NTX, continuous NTX, OGF, or an equivalent volume of sterile water and pulsed with BrdU at designated times. BrdU incorporation in cells treated with continuous NTX was increased by 10 to 37% from 6 to 72 hours compared to control levels (Figure 4.8B). In contrast, DNA synthesis in short term NTX treated cultures was increased by 37% at 6 hours, but decreased by 39, 45, and 32% at 24, 48, and 72 hours, respectively, relative to controls at these time points. The degree of inhibition on DNA synthesis seen with short term NTX was similar at 24, 48, and 72 hours to that recorded in cultures receiving continuous OGF.

Examination of apoptosis (TUNEL) and necrosis (trypan blue staining) at 72 hours in SKOV-3 cells treated with short term NTX, revealed less than 0.1% positive cells for apoptosis and necrosis, and these data were comparable to that obtained with cells subjected to sterile water.

p16 and/or p21 are required for short term NTX's-induced growth inhibition

To evaluate whether the mechanism of short term NTX's effects on cell proliferation requires the p16 and/or p21 CKI pathways, SKOV-3 and OVCAR-3 cells were transfected with siRNAs for scrambled, p16, p21, or both p16 and p21, and treated with short term NTX. At 72 hours, Western blot analysis revealed that p21 expression in SKOV-3 cells transfected with p21 siRNA was reduced up to 67% from untransfected cells; no expression of p16 was recorded (Figure 4.9A). OVCAR-3 cells, however,
transfected with p16 and/or p21 siRNA had significant reductions in expression of p16 (up to 52%) and p21 (up to 54%), relative to untransfected control cultures (Figure 4.9C).

Short term NTX treatment in SKOV-3 cells (which lack p16) reduced cell number from control levels by up to 40% when cells were either untransfected or transfected with scrambled or p16 siRNA (Figure 4.9B). Cell number in cultures with a knockdown of p21 and treated with short term NTX was comparable to control values. A similar pattern of cell alterations was noted with exposure to OGF. Continuous NTX treatment accelerated cell replication by up to 32% regardless of transfection with p16 and/or p21 siRNA.

Using OVCAR-3 cells (which contain p16 and p21) short term NTX repressed cell number up to 43% in untransfected cultures or cells transfected with either scrambled, p16, or p21 siRNA (Figure 4.9D). However, when both p16 and p21 were knocked down in combination, the number of cells at 72 hours in cultures exposed to short term NTX or OGF was comparable to that of sterile water controls. In contrast, continuous exposure to NTX accelerated cell replication up to 63% from control levels, regardless of transfection with p16 and/or p21 siRNA.
Discussion

This study shows for the first time in a tissue culture model that a brief exposure to the opioid antagonists NTX or naloxone suppresses cell proliferation, and that the effects are mediated by opioid peptide-opioid receptor pathways and independent of systemic processes. A number of key observations emerged from this in vitro investigation with respect to opioid antagonist action on growth. First, the duration of opioid receptor blockade determines response in terms of the effects on cell proliferation and not the opioid antagonist itself, explaining what appear to be paradoxical repercussions in our tissue culture studies. Hence, the same concentration of drug utilized for varying periods of time resulted in differing outcomes. Cells subjected to NTX for 6 hours once or every two days, but not daily, depressed cell number, suggesting that a sufficient interval is required for a rebound of opioid action to be observed. However, consistent with previous in vitro reports, continuous blockade of opioid peptides from opioid receptors accelerated cell proliferation, indicating that opioid-receptor interactions function as an inhibitory influence on the cell cycle. Second, opioid peptide-opioid receptor interfacing is tonically active, and critical to the regulation of cell number, as demonstrated by continuous opioid peptide-receptor blockade and escalated cell proliferation. Third, the inhibitory effects of short term NTX exposure on growth are not permanent, indicating that opioid antagonist modulation of cell proliferation is temporary and devoid of toxicity. Fourth, the influence of these agents on growth processes is not related to one specific opioid antagonist, with both NTX and naloxone exhibiting modulatory capabilities under in vitro conditions. Fifth, the inhibition of cell proliferation by opioid antagonists observed in tissue culture is ubiquitous, and was recorded in four diverse cancers. Sixth, opioid antagonist inhibition of growth is not reliant on systemic factors, as the present studies were conducted in a tissue culture setting. Thus, these findings in vitro reveal that opioid antagonist action on growth is not directly related to these agents themselves, but rather is targeted to the interaction of opioid peptides and receptors.

Although it was determined that opioid peptides are tonically active in regulating cell number through a receptor mediated inhibitory pathway, the peptide(s) and receptor(s) involved required identification. Using a variety of opioids, some with high
affinity to classical or non-classical opioid receptors, the pentapeptide OGF was discovered to be the only opioid to alter cell number, a result consonant with previous findings. While the inhibitory action of OGF was similar in magnitude to that of short term NTX, the relationship of OGF to short term NTX and its effects on growth mandated examination. When this regimen of opioid antagonist treatment was tested in the face of neutralization of OGF by antibodies, cells were no longer inhibited by exposure to short term NTX. In fact, cell number was found to be greater than in control cultures, supporting the contention that the opioid peptide involved with short term NTX, OGF, was constitutively expressed and tonically active. To determine which opioid receptor functions in short term NTX action, siRNA technology was used to knockdown the expression of classical and non-classical opioid receptors and these cells were challenged by short term NTX treatment. The results revealed that the loss of only one opioid receptor, OGFr, eliminated the inhibitory effects of a brief exposure to this opioid antagonist. Therefore, these data demonstrate that the repressive action on cell number by exposure to short term NTX is determined by a singular endogenous opioid-opioid receptor pathway: OGF-OGFr.

OGF and OGFr were detected in ovarian cancer cells as reported herein and earlier, indicating that this axis is available for modulation by short term NTX. In examining the repercussions of NTX on the OGF-OGFr axis, we discovered that transcription of neither preproenkephalin (the gene giving rise to OGF) nor OGFr were altered by this opioid antagonist (Figure 4.10A). However, both OGF and OGFr were upregulated in cells exposed to NTX, signifying that opioid receptor blockade results in a compensatory increase at the translational level (Figure 4.10A). As long as NTX is present, the upregulated OGF and OGFr cannot interact, thereby allowing cells to escape the regulatory influence of the OGF-OGFr axis (Figure 4.10B). In the case of short term NTX, the upregulated peptide and receptor can interface and elicit an exaggerated physiological response: inhibition of cell proliferation in the interval when NTX is no longer present (Figure 4.10B). Our findings that either a decrease in OGF (i.e., antibody neutralization) or OGFr (i.e., siRNA) can eliminate the inhibitory effect of short term NTX and increase cell number also indicates that the peptide and the receptor are in a self-regulatory autocrine loop which maintains homeostatic equilibrium.
of cell proliferative processes.

The mechanism of short term NTX's inhibitory action on cell number was found to be related to DNA synthesis, and not to alterations in pathways of cell survival (apoptosis, necrosis). Although the outcome of short term NTX treatment was a reduction in cell number, studies using BrdU as a marker for DNA synthesis showed that brief exposure to NTX resulted in a biphasic response (Figure 4.10A). Thus, DNA synthesis was increased for up to 12 hours after the initiation of short term NTX treatment (i.e., 6 hours after termination of NTX), but was reduced from control levels from 18 hours up to 3 days after initiating opioid antagonist treatment. Moreover, the magnitude of reduction in DNA synthesis in cells exposed to short term NTX was similar to that for OGF. As expected, based on previous findings, DNA synthesis in cells subjected to continuous NTX was increased throughout the 3 day period of drug exposure. Additional experimentation with respect to the molecular target of short term NTX on the cell cycle indicated that p16 and p21 were responsible for NTX's effects. Thus, knockdown of p16 and/or p21 in cancer cells eliminated the inhibitory influence of short term NTX, as well as OGF as reported earlier, thereby providing molecular proof that short term NTX is dependent on CKI pathways.

The results of this study explored the adaptive response to continuous and discontinuous opioid receptor blockade on physiological processes in a tissue culture environment, and have compelling parallels with the action of LDN and HDN in animals, as well as our understanding of the OGF-OGFr axis. These include: i) LDN and HDN lead to an overall decrease and increase, respectively, in DNA synthesis, just as short term and continuous NTX in tissue culture display dissimilar responses on cell replication. ii) LDN and short term NTX have a biphasic effect on DNA synthesis, initially elevating cell proliferation for a brief interval followed by a marked decrease for the remaining period of time. iii) LDN and short term NTX are both dependent on the duration of opioid receptor blockade, and not drug dosage. iv) LDN and short term NTX do not work directly, but rather indirectly through endogenous opioids and opioid receptors. v) Short term NTX and OGF are both dependent on p16 and/or p21 CKI pathways. vi) NTX upregulates OGF and OGFr both under in vitro and in vivo conditions. vii) The effects of LDN and short term NTX are neither toxic
nor related to cell survival. viii) LDN and short term NTX modulate the growth of a wide variety of cells and tissues. Thus, we submit that a tissue culture model of LDN action \textit{in vivo} has been established, allowing an understanding of the mechanism of LDN in a paradigm that is not confounded by systemic interactions.

Our discovery of LDN and HDN with regard to growth in 1983 has led to the transition of opioid antagonists and opioid agonists from the bench to the bedside. LDN has been shown to be non-toxic in a phase I clinical trial, and to have efficacy in improving clinical and inflammatory activity, as well as in promoting mucosal healing, in subjects with active Crohn's disease. With our understanding in the present study, the mechanism of LDN now can be seen to be dependent upon the OGF-OGFr axis, resulting in depression of cell proliferative processes. Particularly important, is that both T and B lymphocyte proliferation have been reported to be suppressed by the OGF-OGFr axis, implying that LDN can serve as a means for modulating autoimmune diseases through this native pathway. In fact, both LDN and OGF prevent and diminish expression of experimental autoimmune encephalomyelitis in preclinical studies, providing novel therapeutic implications for utilization of these agents in patients with multiple sclerosis. OGF also has been found to be non-toxic, and efficacious in the treatment of patients with advanced pancreatic cancer, suggesting that LDN may be effective in utilizing this peptide-receptor axis in the treatment of neoplasia. Taking together the results of the present study in tissue culture showing that the mechanism of short term NTX is dependent on the OGF-OGFr pathway, and the similarities of this \textit{in vitro} model to that of LDN \textit{in vivo}, the action of LDN with regard to cell proliferation can be explained by the targeting of this opioid antagonist to the OGF-OGFr axis. Thus, OGF and LDN function to regulate cell proliferative processes through a common pathway: the OGF-OGFr axis (Figure 4.10).
Figures and Legends

Figure 4.1. Short term and continuous exposure to opioid antagonists and the growth of SKOV-3 cells. (A) Growth curves over 96 hours of cells subjected to short term NTX (either once, every day, or every other day), continuous NTX, or an equivalent volume of sterile (Co) with treatments initiated at time 0. (B) Cell number at 72 hours in cultures exposed daily to NTX for a period of time shorter than 6 hours. (C) Cell number at 72 hours in cultures subjected once to short term naloxone. Compounds and media were replaced daily unless otherwise noted. Data represent means ± s.e.m. from at least 2 aliquots/well and 2 wells/treatment group. Significantly different from Co at respective times by **p<0.01 and ***p<0.001.
Figure 4.2. Short term and continuous exposure to opioid antagonists and the growth of SKOV-3 cells. (A) Cell number at 72 hours in cultures exposed to a variety of opioid related compounds or the opioid receptor antagonist NTX either once for a short term (6 hours) or on a continual (daily) basis. (C) Cell number in cultures treated with NTX or an equivalent volume of sterile water (Co) for 6 hours. At the end of 6 hours, NTX containing media was removed and replaced with media lacking NTX, and cultures were either administered a polyclonal antibody specific for OGF (Anti-OGF) or pre-immune serum (IgG), or were not treated (untreated). Anti-OGF, IgG, compounds, and media were replaced daily unless otherwise noted, and cell number counted at 72 hours. Data represent means ± s.e.m. from at least 2 aliquots/well and 2 wells/treatment group. Significantly different from Co at respective times by **p<0.01 and ***p<0.001.
**Figure 4.3.** OGFr is required for short term NTX and OGF's inhibitory action on the growth of SKOV-3 cells. (A-D) Western blot analysis and quantitative densitometry indicating the specificity and level of (A) MOR, (B) DOR, (C) KOR, and (D) OGFr protein knockdown. Log phase cells were transfected for 24 hours with the indicated siRNAs; protein was isolated 72 hours after the start of transfection. Data represent means ± s.e.m. for the percent of MOR, DOR, KOR, or OGFr relative to actin from 2 independent experiments. (E) Cell number at 72 hours in cultures transfected with the indicated siRNAs. Six hours prior to termination of transfection, cultures were treated with either OGF, NTX, or an equivalent volume of sterile water (Co). At 24 hours, media containing transfection reagents and compounds was replaced with media lacking NTX (short term NTX), or containing NTX (continuous NTX) or OGF. Compounds and media were replaced daily, except for the short term NTX group where this opioid antagonist was only administered once for 6 hours. Values represent means ± s.e.m. from at least 2 aliquots/well and 2 wells/treatment. Significantly different from untransfected Co cultures at **p<0.01 or ***p<0.001.
Figure 4.4. The effects of short term and continuous NTX treatments on PPE mRNA and OGF. Cultures were administered NTX either once for 6 hours or continuously, or subjected to an equivalent volume of sterile water (Co). Media and compounds were replaced daily unless otherwise indicated. (A) Expression of PPE mRNA. RNA was isolated at the indicated times, reverse transcribed, and the generated cDNA was amplified by PCR with primers for PPE and β-globulin. PCR products were separated on an agarose gel, stained with ethidium bromide, and analyzed by densitometry. Data represent means ± s.e.m. for the percent of the ratio of PPE relative to β-globulin from 2 wells/treatment group in 3 independent experiments. (B) Expression of cellular OGF. Photomicrographs of cells stained with a polyclonal antibody to OGF, and semiquantitative densitometry of staining intensity (mean gray value). Inset = secondary antibody only. Bar = 10 μm. Data represent means ± s.e.m. for at least 100 cells/coverglass and 3 coverglasses/treatment group. (C) Levels of secreted OGF in cultures treated with NTX in serum-free media either once for a short duration (6 hours) or continuously, or subjected to an equivalent volume of sterile water (Co). At 6 hours, media containing compound was replaced with serum-free media; media was not changed after this time point. The continuous NTX group received this opioid antagonist on a daily basis. Media was collected at indicated times and subjected to radioimmunoassay. Data represent means ± s.e.m. from at least 2 wells/treatment group assayed in duplicate. Significantly different from Co at *p<0.05, **p<0.01, and ***p<0.001, and from continuous NTX at +++p<0.001.
SKOV-3

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Co

Continuous NTX

Short term NTX

24 hours 72 hours

\[\text{Co} \, \text{Short term NTX} \, \text{Continuous NTX}\]

C

\[\text{Control} \, \text{Short term NTX} \, \text{Continuous NTX}\]

OGF (pg / 10^5 cells)
**Figure 4.5.** The effects of short term and continuous NTX treatments on OGFr mRNA and protein. Cultures were treated with NTX either once for a short term (6 hours) or continuously, or with an equivalent volume of sterile water (Co). (A) OGFr mRNA levels measured by Northern blot and densitometric analysis at the indicated times. Data represent means ± s.e.m. for the percent of OGFr relative to GAPDH from 2 independent experiments. (B) OGFr expression assessed by immunohistochemistry. Photomicrographs of cells stained with a polyclonal antibody to OGFr and evaluated by semiquantitative densitometry (mean gray value). Bar = 10 μm. Data represent means ± s.e.m. for 100 cells/coverglass and 3 coverglasses/treatment group. (C) OGFr expression measured by Western blot analysis. Total proteins were isolated at the indicated times, probed with antibodies specific to OGFr or actin, and measured by quantitative densitometry. Data represent means ± s.e.m. of the percent of OGFr relative to actin from 2 independent experiments. (D) Representative saturation isotherms and Scatchard plots of specific binding of [³H]-[Met⁵]-enkephalin to nuclear homogenates of SKOV-3 cells at 72 hours. Means ± s.e.m. for binding affinity (Kₐ) and binding capacity (Bₘₐₓ) determined from at least 3 independent assays performed in duplicate. Significantly different from Co at *p<0.05, **p<0.01, and ***p<0.001, and from continuous NTX at +p<0.05.
Figure 4.6. The effects of NTX and OGF are ubiquitous. Cell number at 72 hours in OVCAR-3, SCC-1, MiaPaCa-2, and HCT-116 cells exposed to NTX for either a short or continuous duration, OGF, or an equivalent volume of sterile water (Co). Data represent means ± s.e.m. Significantly different from Co at **p<0.01 or ***p<0.001.
Figure 4.7. The combination of short term NTX followed by daily OGF treatment provides an additive inhibitory effect on reducing cell number in SKOV-3 and OVCAR-3 cultures. Cell number at 72 hours in cultures treated with NTX once for 6 hours (short term NTX), OGF, the combination of short term NTX followed by OGF, or an equivalent volume of sterile water (Co). Media was replaced daily, as were compounds except for the short term NTX group. Values represent means ± s.e.m. for at least 2 aliquots/well and 2 wells/treatment group. Significantly different from Co at ***p<0.001, from OGF at ^^p<0.01 and ^^^p<0.001, and from short term NTX at +++p<0.001.
Figure 4.8. Effect of short term NTX, continuous NTX, or OGF on DNA synthesis. (A) DNA synthesis in cells treated with one 6 hour exposure to NTX (short term NTX) or an equivalent volume of sterile water (Co), and incubated with BrdU 3 hours prior to fixation at the indicated times. Treatments were initiated so that all groups were harvested at 72 hours. (B) DNA synthesis in cells treated with one 6 hour application of NTX (short term NTX), continuous NTX, OGF, or an equivalent volume of sterile water (Co), and incubated with BrdU 3 hours prior to fixation at the indicated times. Data represent means ± s.e.m. Significantly different from Co by *p<0.05, **p<0.01, and ***p<0.001.
**Figure 4.9.** Inhibition of cell proliferation by short term NTX treatment requires p16/p21 pathways. (A, C) Western blot analysis demonstrating the specificity and knockdown of p16/p21 in (A) SKOV-3 and (C) OVCAR-3 cells. Cells were transfected for 24 hours with p16/p21 or scrambled siRNAs, total proteins isolated 72 hours after the start of transfection, preparations probed with antibodies specific to p16, p21, or actin, and blots measured by quantitative densitometry. Data represent means ± s.e.m. for the percent of p16 or p21 relative to actin from 2 independent experiments. (B, D) Cell number at 72 hours in (B) SKOV-3 and (D) OVCAR-3 cells transfected with the indicated siRNAs and treated with NTX either for a short term or continuously, OGF, or an equivalent volume of sterile water (Co). Values represent means ± s.e.m. for 2 aliquots/well and 2 wells/treatment group. Significantly different from untransfected Co cultures at **p<0.01 and ***p<0.001.
Figure 4.10. Schematic representation of the effects of NTX administered for either a short or continuous duration on the OGF-OGFr axis. (A) Effects of a single 6 hour application of short term NTX (dotted line), continuous NTX (dashed line), or equivalent volume of sterile water (solid line) on the levels of PPE mRNA, cellular OGF, secreted OGF, OGFr mRNA, OGFr protein, cell number, and DNA synthesis. (B) Schematic illustrating the action of short term NTX, continuous NTX, or exogenous OGF in the regulation of cell proliferative events.
A

**Short term NTX**
- PPE mRNA
- Cellular OGF
- Secreted OGF
- OGFr mRNA
- OGFr protein
- Cell number
- DNA synthesis

**Continuous NTX**

![Graph showing time-course of expression levels for different parameters under short and continuous NTX](image)

B

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References


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Chapter 5: LDN *In Vivo* and in Combination with Chemotherapy

**Rationale**

Experiments have documented that short term opioid receptor antagonism with NTX (or the equivalent of LDN) inhibits human ovarian cancer cell proliferation. The question can be raised as to whether upregulation of the OGF-OGFr axis by LDN functions *in vivo* to regulate ovarian tumor growth. Our knowledge that LDN functions to mediate its inhibitory effects on growth through an upregulation of the OGF-OGFr axis, which targets cell proliferation without inducing apoptosis, prompts an examination on whether LDN can be utilized in combination with the standard of care chemotherapies to provide an additive inhibitory effect on ovarian cancer. Using a subcutaneous xenograft model where tumor growth can easily be tracked over time, the goal of this next aim was to establish whether upregulation of the OGF-OGFr axis by LDN biotherapy, administered alone and in combination with the standard of care chemotherapies taxol or cisplatin, regulates cell proliferation and tumor growth of ovarian cancer, as well as ascertain the mechanism of its action alone and in combination treatments.
Low dose naltrexone (LDN) suppresses ovarian cancer and exhibits enhanced inhibition in combination with cisplatin

Accepted for publication in *Experimental Biology and Medicine*

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Abstract

Ovarian cancer is the leading cause of death from gynecological malignancies. Although initial therapeutic modalities are successful, 65% of these women relapse with only palliative treatments available thereafter. Endogenous opioids repress the proliferation of human ovarian cancer cells in vitro, and do so in a receptor mediated manner. The present study examined whether modulation of opioid systems by the opioid antagonist naltrexone (NTX), alone or in combination with standard of care therapies (taxol/paclitaxel, cisplatin), alters human ovarian cancer cell proliferation in tissue culture and tumor progression in mice. Administration of NTX for 6 h every two days, but not continuously, reduced DNA synthesis and cell replication from vehicle treated controls in tissue culture. Moreover, brief exposure to NTX in combination with taxol or cisplatin had an enhanced anticancer action. Mice with established ovarian tumors and treated with a low dosage of NTX (LDN), which invokes a short period of opioid receptor blockade, repressed tumor progression in a non-toxic fashion by reducing DNA synthesis and angiogenesis but not altering cell survival. The combination of LDN with cisplatin, but not taxol, resulted in an additive inhibitory effect on tumorigenesis with enhanced depression of DNA synthesis and angiogenesis. LDN combined with cisplatin alleviated the toxicity (e.g., weight loss) associated with cisplatin. LDN treatment upregulated the expression of the opioid growth factor (OGF, chemical term ([Met\(^5\)]-enkephalin) and its receptor, OGFr. Previous tissue culture studies have reported that OGF is the only opioid peptide regulating proliferation of ovarian cancer cells, with OGF action mediated by OGFr. Thus, the common denominator of intermittent opioid receptor blockade by short term NTX or LDN on ovarian cancer proliferation and tumorigenesis recorded herein appears to be related to the OGF-OGFr axis. These preclinical data may offer a non-toxic and efficacious pathway-related treatment that can benefit patients with ovarian cancer.
Introduction

Ovarian cancer is the 5th leading cause of cancer mortality among women in the United States and the major cause of death from gynecological malignancies. More than 75% of women are diagnosed with ovarian cancer in advanced stages. Ninety percent of these neoplasias are epithelial in origin. Although the initial clinical response to cytoreductive surgery and adjuvant chemotherapy is excellent, nearly 65% of advanced-staged patients relapse within 2 years and, upon recurrence, all subsequent treatments are palliative. The cellular and molecular events involved in the pathogenesis of this deadly neoplasm need to be defined, as major improvements in the prognosis and treatment of ovarian cancer patients will necessitate novel therapies that target biological pathways.

Endogenous opioids and opioid receptors have been shown to have tonically active growth regulatory properties in neoplasia, including human ovarian cancer. Opioid antagonist modulation of endogenous opioid systems has been used to decipher the function of opioid peptide-opioid receptor interactions in a number of biological processes and diseases including cancer. Naltrexone (NTX) is a general opioid receptor antagonist that is devoid of intrinsic activity and blocks endogenous opioids from opioid receptors. This opioid receptor blockade leads to an upregulation in the production of endogenous opioid peptides and opioid receptors. Continuous opioid receptor blockade, achieved using a daily high dose of NTX (HDN) (e.g., 10 mg/kg in mice) or administration of a low dose of naltrexone (LDN) (e.g., 0.1 mg/kg in mice) multiple times each day, does not permit the interfacing of the upregulated opioids and receptors. Such a constant opioid receptor blockade results in repercussions such as an acceleration in DNA synthesis and tumor progression. However, pharmacokinetic, nociceptive, and functional studies have shown that blockade of opioid peptides from opioid receptors for a short period each day (4 to 6 h), using a daily administration of LDN, provides an 18 to 20 h window wherein the elevated levels of endogenous opioids and opioid receptors can interact to elicit a response (e.g. depression of DNA synthesis, inhibition of tumorigenesis). One particular endogenous opioid peptide-opioid receptor system involved in growth regulation that NTX has been demonstrated to upregulate is the opioid growth
factor (OGF) and its receptor, OGFr. OGF, chemically termed [Met]-enkephalin, is a constitutively expressed native opioid peptide that is autocrine produced and secreted. OGF interacts with OGFr (a non-classical opioid receptor) to delay the G1/S phase of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways, and inhibits cell proliferation in normal and neoplastic cells, including ovarian cancer. The OGF-OGFr axis has been shown to be present in human ovarian cancer, with OGFr RNA, protein and binding activity documented in these cells in vitro, and OGF detected by radioimmunoassay in surgical samples taken from human ovarian neoplasms. Studies in tissue culture have documented that OGF is the only endogenous opioid peptide which regulates ovarian cancer cell proliferation, and that the inhibitory action of OGF is mediated by OGFr. An increase in OGF-OGFr activity in human ovarian cancer cells in tissue culture by the addition of exogenous OGF has been reported to markedly suppress cell proliferation in a non toxic manner by targeting the CKI pathways. Moreover, continuous intervention of opioid peptide-opioid receptor interaction with the opioid antagonist NTX accelerates cell proliferation.

Given that the OGF-OGFr axis is present and functions in an inhibitory manner in human ovarian cancer cells in tissue culture, and that persistent opioid receptor blockade by NTX increases DNA synthesis and cell number, this raises the question of whether modulation of the OGF-OGFr system by intermittent opioid receptor blockade (e.g., LDN) can alter the progression of this gynecological cancer. In addition, this study inquired as to whether LDN could be combined with either of two standard of care therapies, taxol (paclitaxel) or cisplatin, to provide an added inhibitory effect on ovarian tumorigenesis. To initially address these questions, we have developed a tissue culture model that exposes ovarian cancer cells to NTX for a short period of time, resulting in suppression of DNA synthesis and cell proliferation. This in vitro model using short term NTX exposure has compelling parallels with LDN in vivo, and allowed direct examination of the repercussions of combining a brief duration of opioid receptor blockade with taxol or cisplatin on ovarian cancer without confounding systemic influences. To gain the full perspective of the biological significance of modulating the OGF-OGFr axis with LDN, we evaluated the effects of LDN alone and in combination with chemotherapy in mice with xenografts of established human ovarian cancer.
Material and methods

Cell culture

The human ovarian cancer cell line SKOV-3 was obtained from The American Type Culture Collection (Manassas, VA). Cells were grown in a humidified atmosphere of 5% CO$_2$/95% air at 37°C in RPMI medium supplemented with 1.2% sodium bicarbonate, 10% fetal calf serum, and antibiotics (5,000 units/mL penicillin, 5 µg/mL streptomycin, and 10 mg/mL neomycin).

Growth assays

Cells were plated and counted 24 h later (time 0) to determine seeding efficiency. Cultures were treated with NTX ($10^{-5}$ M), taxol ($10^{-9}$ or $10^{-10}$ M), cisplatin (0.01 or 0.001 µg/mL), NTX ($10^{-5}$ M) and taxol ($10^{-9}$ or $10^{-10}$ M), NTX ($10^{-5}$ M) and cisplatin (0.01 or 0.001 µg/mL), or an equivalent volume of sterile water. At the end of 6 h, the media containing compound(s) was removed and replaced with media either lacking NTX (short term NTX treatment), or containing NTX (continuous NTX treatment), taxol, or cisplatin. Media and compounds were replaced on a daily basis except for the short term NTX group, where treatment was administered every 48 h. In the reversal studies, some cultures had media replaced without compound at 48 h. Taxol was dissolved in DMSO ($10^{-2}$ M) and further diluted in sterile water; all other compounds were prepared in sterile water, and dilutions represent final concentrations. An equivalent volume of vehicle was added to control (Co) wells. Cells were harvested at designated times, stained with trypan blue, and counted with a hemacytometer. At least 2 aliquots/well and 2 wells/treatment/timepoint were sampled.

Animals, tumor cell implantation, and tumor growth

Four week-old athymic nu/nu female mice, purchased from The Charles River Laboratory (Wilmington, MA), were housed in pathogen-free isolator ventilated cages in a controlled-temperature room (22-25°C) with a 12-12 h light/dark cycle (lights on 0700-1900). Sterile rodent diet (Teklad, Indianapolis, IN) and water were available ad libitum. All procedures were approved by the IACUC Committee of The Pennsylvania State University College of Medicine, and conformed to the guidelines established by the NIH. Following a 48 h acclimation period, unanaesthetized mice were injected subcutaneously (s.c.) with SKOV-3 cells ($4 \times 10^6$/mouse) into the right scapula region.
Mice were weighed 3 times/week, observed daily for initial appearance of tumors, and tumors were measured 3 times/week using vernier calipers. Volume was calculated using the formula $l \times w^2 \times \pi/6$ where length ($l$) is the longest dimension, and width ($w$) is the dimension perpendicular to the length.\textsuperscript{35}

**Drug treatment**

Beginning on the day tumors became visible (day 0), six groups of mice ($n = 10$) were randomly assigned to receive intraperitoneal (i.p.) injections of LDN (0.1 mg/kg, daily), taxol (3 mg/kg, days 0, 7, 14, 21, 28, and 35), cisplatin (4 mg/kg, days 0 and 7), LDN and taxol, LDN and cisplatin, or an equivalent volume of saline (daily). These dosages were selected based on published reports.\textsuperscript{8, 10, 21, 22, 36, 37} To ensure that all mice were injected an equivalent number of times, animals not assigned to receive treatment on a given day were administered saline. In groups receiving combined therapy, LDN was administered first. Taxol was dissolved in DMSO ($10^{-2}$ M) and further diluted in saline, while LDN and cisplatin were dissolved in saline; all drugs were prepared weekly.

**Termination day measurements**

According to the IACUC guidelines, the study was terminated when tumors became ulcerated or grew to 2 cm in diameter. All mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and cervical dislocation 35 days following initiation of treatments. For examination of DNA synthesis in tumors, a subset of mice from each group were injected i.p. twice with 100 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO) at 6 and 3 h prior to euthanasia. Tumors and spleens were removed and weighed, and the lymph nodes, liver, and spleen examined for metastases. Tumor tissue was assessed for expression of OGF and OGFr, cell survival, angiogenesis, and DNA synthesis.

**Semiquantitative immunohistochemistry**

Immunohistochemistry was utilized to evaluate the presence and relative level of OGF and OGFr in tumor tissue following published procedures.\textsuperscript{5, 38, 39} Tumors were excised, frozen in chilled isopentane, sectioned at 10 $\mu$m, fixed, permeabilized, and stained with antibodies to OGF and OGFr that were generated and characterized in our laboratory.\textsuperscript{40} Images were taken at the same exposure time with care not to
Photobleach samples. A random sample of at least 10 fields/section, 2 sections/tumor, and 3 tumors/group were evaluated. Controls were incubated with secondary antibodies only.

**Protein isolation and Western blotting**

Expression of OGFr was evaluated in tumors by Western blotting following published procedures. Briefly, tissue was homogenized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN). Protein (60 μg) was subjected to 15% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to OGFr (1:200). Optical densities were normalized to β-actin (1:5000, Sigma-Aldrich), and the percent change in expression was calculated by dividing the normalized values of experimental samples to that of saline controls. Means and SE were determined from 2 independent experiments.

**OGFr binding assays**

Tumors were assayed for OGFr binding using custom synthesized [³H]-[Met⁵]-enkephalin (Perkin Elmer, Waltham, MA; 52.7 Ci/mmol) following published procedures. Saturation binding isotherms were generated using GraphPad Prism software (La Jolla, CA), and independent assays were performed at least 3 times.

**Mechanism of growth inhibition: DNA synthesis, angiogenesis, apoptosis, and necrosis**

Cells were assayed for DNA synthesis, necrosis, and apoptosis, whereas tumor tissue was evaluated for DNA synthesis, apoptosis, and angiogenesis. To measure DNA synthesis, cells were treated with 30 μM BrdU for 3 h prior to fixation, while tumors from mice receiving BrdU on the day of sacrifice were fixed in formalin overnight, processed in paraffin, and sectioned at 10 μm. Preparations were processed with antibodies to BrdU (1:200, Invitrogen, Carlsbad, CA) to assess DNA synthesis, stained with hematoxylin/eosin to evaluate endothelial cell-lined vessels containing red blood cells, or processed for TUNEL according to the manufacturer's instruction to measure apoptosis (Trevigen, Gaithersburg, MD). For cells in tissue culture, the proportion of BrdU or TUNEL positive cells was determined for at least 500 cells on 2 coverglasses/treatment group. For tumors, the proportion of BrdU positive cells, number of TUNEL positive cells, and blood vessel density were determined from at
least 10 random fields around the periphery of each tumor, with at least 2 sections/tumor, and 2 tumors/treatment group evaluated. BrdU and TUNEL positive cells were counted in a 0.003 mm$^2$ area, while blood vessel density was determined in a 0.16 mm$^2$ area.

**Chemicals**

NTX was obtained from Sigma-Aldrich. Cisplatin was purchased from Alexis Biochemicals (Lausen, Switzerland), and taxol was obtained from Toronto Research Chemicals (North York, ON).

**Statistical analysis**

All data were analyzed using one way analysis of variance (ANOVA), with subsequent comparisons made using Newman–Keuls tests (Graph Pad Prism Software). In some cases, data were evaluated using unpaired t-tests. P values <0.05 were considered to be significant.
Results

Combination of short term NTX treatment with taxol or cisplatin provides an additive inhibitory effect on ovarian cancer cell number: *In vitro* studies

To establish the effects of a short term treatment of NTX on the growth of human ovarian cancer cells, SKOV-3 cultures were treated with $10^{-5}$ M NTX for 6 h every other day; this dosage and regimen inhibited the proliferation of SKOV-3 cells by 28 to 42% between 48 and 120 h. However, in cultures treated with continuous NTX, cell number was increased 19 to 31% between 48 and 120 h, relative to sterile water treated cells, and increased 69 to 109% between 48 and 120 h, relative to cultures treated with short term NTX (Figures 5.1A, B).

To ascertain the repercussions of combining a short term NTX treatment with taxol, cells were exposed to these agents alone and in combination. The dosages of taxol ($10^{-9}$ and $10^{-10}$ M) were selected based on preliminary experiments that revealed no logarithmic growth at higher concentrations. Taxol at either concentration inhibited the number of SKOV-3 cells by 21 to 67% (Figure 5.1A). In cultures receiving short term NTX (every 48 h) with taxol (at either concentration), cell number was reduced 36 to 61% compared to cells exposed to short term NTX alone, and reduced 19 to 31% relative to cells treated with either concentration of taxol alone. At all time points evaluated over a 120 h period, an equivalent number of cells was noted in cultures receiving a combination of short term NTX and taxol ($10^{-10}$ M) compared to those receiving the higher concentration of taxol ($10^{-9}$ M) alone.

To investigate the consequence of coupling a short term NTX treatment with cisplatin, log phase cultures were subjected to these agents alone and in combination. The dosages of cisplatin (0.01 and 0.001 μg/mL) were chosen in view of preliminary experiments that revealed no logarithmic growth at higher concentrations. Cisplatin at either concentration suppressed the number of ovarian cancer cells by 23 to 51% (Figure 5.1B). In cultures receiving a short exposure to NTX every 48 h in combination with cisplatin at either concentration, cell number was reduced 21 to 42% compared to cells treated with this same regimen of NTX alone, and 23 to 32% relative to cells subjected to either concentration of cisplatin alone. At all time points evaluated, an equivalent number of cells was detected in cultures receiving short term NTX and
cisplatin (0.001 μg/mL) compared to those receiving the higher concentration of cisplatin (0.01 μg/mL) alone.

**The growth effects of short term exposure to NTX, but not to continuous treatment with taxol or cisplatin, are reversible**

To study whether the effects of NTX given for a short or continuous duration, as well as either taxol or cisplatin, on growth could be reversed by withdrawing cells from drug exposure, SKOV-3 cultures were treated with short term NTX (10⁻⁵ M) every 48 h, continuous NTX (10⁻⁵ M), taxol (10⁻⁹ or 10⁻¹⁰ M), cisplatin (0.01 or 0.001 μg/mL), or an equivalent volume of sterile water. At 48 h, media and compounds were removed from all cultures and replaced with either media containing compounds or media lacking these agents (i.e., reversal). At 96 and 120 h, the short term NTX-reversal group had 8 and 36% more cells, respectively, than in the group continuing to receive 6 h of exposure to NTX every 48 h (Figures 5.1C-F). However, cultures in the continuous NTX-reversal group had 9 and 20% less cells at 96 and 120 h, respectively, than cells maintained on NTX. The taxol and cisplatin reversal groups did not differ from cultures continuing to be treated with taxol or cisplatin, respectively.

**Mechanism of enhanced growth inhibition by the combination of short term NTX with taxol or cisplatin: *In vitro* studies**

Examination of apoptosis and necrosis in SKOV-3 cells treated with NTX (10⁻⁵ M) for either a short or long term duration, taxol (10⁻⁹ or 10⁻¹⁰ M), cisplatin (0.01 or 0.001 μg/mL), or a combination of short term NTX with taxol or cisplatin (at either concentrations of these agents), revealed less than 0.1% positive cells for apoptosis or necrosis; these data were comparable to that obtained with cells subjected to sterile water (data not shown).

In regard to DNA synthesis, cells treated with NTX for a short term had 38% fewer cells labeled with BrdU than sterile water controls (Figures 5.2A, B). In contrast, cells subjected to NTX continuously had a 42% increase in BrdU labeling from cultures subjected to water. The combination of short term exposure to NTX with taxol (at either concentration) reduced BrdU labeling by 47 to 49% from the group receiving taxol alone (Figure 5.2A). The coupling of NTX given for a short duration with taxol at either concentration suppressed DNA synthesis 39 to 47% from cells receiving NTX alone.
Cultures receiving a short term exposure to NTX in combination with cisplatin (at either concentration) had 38 to 44% decreased BrdU labeling indexes in contrast to cultures receiving only NTX, and 27 to 52% reduced labeling indexes relative to cultures receiving cisplatin alone (Figure 5.2B).

**LDN inhibits established ovarian cancer, and has an additive inhibitory effect on tumor progression in combination with cisplatin**

Beginning 2 days after initiation of treatments and persisting throughout the study, tumor volumes in mice with established s.c. ovarian xenografts were reduced by treatment with LDN (21 to 48%), taxol (21 to 54%), cisplatin (24 to 54%), LDN and taxol (39 to 60%), or LDN and cisplatin (16 to 60%) compared to control animals receiving saline (Figures 5.3A, B). The reductions in tumor volumes in mice subjected to LDN, taxol, cisplatin, or the combination of LDN and taxol were comparable. However, animals administered both LDN and cisplatin had tumor volumes that were reduced 9 to 30% from mice treated with LDN alone beginning on day 14, and were decreased 15 to 37% from mice receiving cisplatin alone beginning on day 16 (Figure 5.3B).

On the day of termination (day 35), mice from all treatment groups displayed a visible reduction in tumor size (Figure 5.3C) compared to controls subjected to saline, with decreases in both tumor volume (43 to 55%, Figure 5.3D) and tumor weight (28 to 46%, Figure 5.3E) recorded. The reductions in tumor volume and weight in mice exposed to the combination of LDN and taxol were comparable to those in animals subjected to LDN or taxol alone (Figures 5.3D, E). However, relative to mice treated with cisplatin alone, the combination of LDN and cisplatin depressed tumor volumes and tumor weights by 26 and 25%, respectively. Mice receiving the combination of LDN and cisplatin had terminal tumor volume and weight that were equivalent to mice treated with LDN alone.

**Effects of treatments on body weight and gross observations**

Although all mice weighed approximately 18 to 20 g at the beginning of the experiment (Figure 5.4), mice receiving cisplatin on days 0 and 7 had a 7 to 12% reduction in body weight compared to saline administered controls on days 2, 9, and 11 of the study. Mice receiving the combination of LDN and cisplatin also were reduced from saline treated controls in body weight (8%) on day 2 of the study. However,
cohorts exposed to both LDN and cisplatin were comparable in body weight to saline administered controls on day 9 and 11 of the study. Body weights of animals administered LDN, taxol, or the combination of LDN and taxol, were similar throughout the study to saline controls (Figure 5.4).

Spleen weights on the day of termination did not differ between any group of mice (data not shown), and behavioral abnormalities were not evident. Metastases or lesions were not detected in mice from any group.

**Mechanism of enhanced growth inhibition by LDN with cisplatin: Effects on apoptosis, DNA synthesis, and angiogenesis**

Examination of apoptosis by TUNEL assay revealed similar levels of programmed cell death in tumors taken from mice treated with LDN or saline (Figure 5.5A). However, animals treated with taxol or cisplatin, either alone or in combination with LDN, had approximately a 3-fold greater number of apoptotic cells in tumors than that of saline administered controls.

With respect to cells in tumors undergoing DNA synthesis, a reduction of 42 to 57% was noted in all treatment groups compared to mice injected with saline (Figure 5.5B). The BrdU labeling index in tumors from animals receiving the combination of LDN and taxol was comparable to cohorts subjected to either LDN or taxol alone, while the BrdU labeling index in tumors from animals receiving both LDN and cisplatin was reduced 24% compared to subjects treated with cisplatin alone. Levels of DNA synthesis in tumors were similar in mice treated with only LDN or the combination of LDN and cisplatin.

With respect to the density of blood vessels in tumors, blood vessel density was reduced 52 to 73% in all treatment groups relative to animals exposed to saline (Figure 5.5C). Blood vessel density in tumors from mice receiving the combination of LDN and taxol was comparable to cohorts treated with LDN or taxol alone. In contrast, blood vessel density was decreased 42 to 44% in subjects treated with the combination of LDN and cisplatin compared to animals administered LDN or cisplatin alone.

**The presence and expression of OGF and OGFr in ovarian cancer xenografts**

To evaluate OGF distribution and relative expression levels in xenografts of ovarian tumors, semiquantitative immunohistochemistry was performed. OGF was
visible in the cytoplasm and a speckling of immunoreactivity was often noted in cell nuclei (Figure 5.6A). Tumors processed with only secondary antibody showed no staining (Figure 5.6A inset). OGF distribution did not differ between groups. However, OGF immunofluorescence (mean gray value) in mice treated with LDN, both LDN and taxol, or the combination of LDN with cisplatin was increased 33 to 39% relative to animals administered saline (Figure 5.6B). Comparable levels of OGF immunofluorescence were noted in tumors from mice treated with saline, taxol, or cisplatin.

To examine OGFr distribution and relative expression, immunohistochemistry, Western blotting, and receptor binding assays were performed on xenografts. The cellular location of OGFr was similar in all groups, with immunoreactivity detected in the cytoplasm and nucleus (Figure 5.6C). Tumors processed with only secondary antibody showed no staining (Figure 5.6C inset). Relative to saline administered controls, OGFr expression in mice treated with LDN, LDN and taxol, or LDN and cisplatin was increased 46 to 61% using semiquantitative immunohistochemistry (Figure 5.6D). Further evaluation of OGFr expression using Western blotting showed that mice treated with LDN had an 87% increase in OGFr expression in their tumors compared to saline administered controls (Figures 5.6E, F).

Receptor binding assays indicated specific and saturable binding for OGFr in tumors of all groups, with a one site model of binding recorded (Figure 5.6G). Binding capacity ($B_{\text{max}}$) values were markedly increased (112 to 136%) in mice treated with LDN, LDN and taxol, or LDN and cisplatin, compared to control animals receiving saline. However, binding affinity ($K_d$) for OGFr did not differ among treatment groups and ranged from 2.7 to 6.2 nM (data not shown).
Discussion

The present study demonstrates for the first time that exposure to NTX for a short duration suppresses cell proliferation and DNA synthesis \textit{in vitro}. This is in distinct contrast to a long term continuous exposure to NTX which accelerates cell proliferation and DNA synthesis of human ovarian cancer cells, a result reported previously.\textsuperscript{5,6} The effects of a short term treatment with NTX on proliferation of ovarian cancer cells was comparable to that of the reductions recorded herein and elsewhere with taxol or cisplatin.\textsuperscript{45,46} When NTX in a regimen of short term exposure was combined with taxol or cisplatin under \textit{in vitro} conditions, the effects on cell replication were greater than with the individual drugs. Indeed, the effects of a $10^{-9}$ M or 0.01 μg/mL concentration of taxol or cisplatin, respectively, could be achieved with a 10-fold lower concentration of either agent when combined with a short term treatment with NTX. The inhibitory influence on cell proliferation by short term treatment with NTX was reversible, with cell number having a trajectory returning to control levels upon withdrawal of this opioid antagonist. In contrast, discontinuation of taxol or cisplatin treatment by providing fresh media without either drug did not change cell kinetics, with these cultures resembling those that continued to receive either drug. These results show that the combination of two treatment modalities, NTX given for a short duration and a chemotherapeutic agent, act in an additive fashion to impede the growth of human ovarian cancer cells in tissue culture.

The results of this study make the seminal observation that intermittent opioid receptor antagonism, achieved by a daily exposure to LDN, markedly impedes the progression of human ovarian tumorigenesis. Treatment with LDN resulted in reductions in both tumor volume and weight that were approximately one-third of those for tumor-bearing animals injected with saline. Moreover, the magnitude of effects of LDN on tumorigenicity were equal to that recorded with either of two standard of care agents: taxol and cisplatin. When LDN was combined with cisplatin, but not with taxol, even greater anti-tumor activity than either agent alone was recorded. This is in contrast to the results obtained \textit{in vitro}, wherein a short term exposure to NTX could be combined with either taxol or cisplatin for an enhanced inhibitory effect on growth. This is not the first time, however, that a discrepancy in the sensitivity to a chemotherapeutic
agent has been observed when testing drugs under in vitro and in vivo conditions. In summary, these results provide evidence that LDN has a potent antitumor effect on ovarian carcinogenesis, and reveal that a combination of biotherapeutic modulation with LDN and chemotherapy with cisplatin have a cooperative effect in retarding the growth of this lethal disease.

Although both LDN and/or taxol in the concentrations and regimens used in this study were not overtly toxic to mice with xenografts of ovarian cancer, animals subjected to cisplatin alone had notable reductions in body weight on several days in the study. This systemic toxicity from cisplatin was diminished by simultaneous administration with LDN, indicating that this regimen with an opioid antagonist has the capacity to protect against toxicological insults. The amelioration of cisplatin induced toxicity by LDN, however, was not accompanied by a diminution of the antitumor action of cisplatin. In fact, the combination of LDN and cisplatin had an effect on tumor growth (i.e. weight, volume) that exceeded cisplatin alone. The mechanism of protection afforded by LDN against cisplatin toxicity is unknown. The alleviation of toxicity of one agent by the administration of another drug, however, is not without precedence. The finding of protection afforded by LDN from the side effects of cisplatin may allow higher doses of cisplatin to be administered to improve the therapeutic efficacy of this agent. This may be advantageous, as the success of chemotherapy is often limited by resistance of cancer cells as well as toxicity, and the possibility of increasing the concentration of drugs without an accompanying increase in adverse events could be extremely beneficial.

The mechanism for enhanced growth inhibition of SKOV-3 cells in tissue culture by a combination of short term NTX with either taxol or cisplatin was related to DNA synthesis but was not associated with induction of apoptosis or necrosis, at least at the low dosages of taxol and cisplatin used herein. Under in vivo conditions, however, the combination of LDN and cisplatin, but not the coupling of LDN and taxol, had an additive effect on tumorigenic events. Both taxol and cisplatin induced apoptosis, depressed cell proliferation, and reduced the number of blood vessels. LDN, on the other hand, altered cell proliferation and angiogenesis, but did not influence apoptosis. The mechanism of the enhanced effect of LDN and cisplatin on ovarian cancer appears to be related to the
number of cells undergoing DNA synthesis, as well the density of blood vessels, when these agents are combined. The lack of enhanced activity of a combination of LDN and taxol on tumorigenesis appears to be correlated with a failure of these agents to have an additive action on cell proliferation or angiogenesis. The effects of these agents are consistent with previous observations. Taxol and cisplatin are well known to induce cell death in a cell-phase specific manner at G2/M through binding to and stabilizing microtubules, and binding to DNA and nuclear proteins to form intra and interstrand crosslinks, respectively. The demonstration that LDN inhibits DNA synthesis to reduce tumorigenesis is also consistent with a previous study where mice transplanted with murine neuroblastoma were treated with LDN. In that study, DNA synthesis was initially increased during the period of opioid receptor blockade (4 to 6 h), but was markedly depressed in the subsequent 18 to 20 h interval when NTX was no longer present, resulting in a net inhibition of cell proliferation and tumorigenic events. Thus, the individual effects of biotherapy and chemotherapy can be enhanced by combining agents that target similar and differing fundamental biological processes.

In all previous studies investigating the effects of an intermittent opioid receptor blockade with LDN on carcinogenesis, the paradigm used was to initiate administration of opioid antagonist concomitant with tumor cell inoculation. These reports concluded that the effects of LDN were pronounced as to altering the early events of tumor development (latency to initiation) without affecting events subsequent to appearance (tumor growth). The present study, however, reveals for the first time that LDN functions to inhibit ovarian cancer progression in mice with established tumors. These results reveal that LDN not only can influence early tumorigenic events, but can exert a potent action on an established cancer. With these observations in mind, it may be conjectured that LDN can be used as an antitumor agent in ovarian cancer prior to tumor expression and serve as a prophylactic therapy. Moreover, our present findings would suggest that patients with established disease or following tumor resection could benefit from LDN biotherapy alone or in combination with standard of care drugs.

A number of lines of evidence from previous reports, as well as the present investigation, suggest that the opioid peptide-opioid receptor system involved with the
inhibitory action of both short term exposure to NTX *in vitro*, and intermittent opioid receptor blockade with LDN *in vivo*, is the OGF-OGFr axis. First, amongst a panel of natural and synthetic opioid peptides, many specific for the classic µ, δ, or κ opioid receptors, OGF was the singular opioid peptide with growth inhibitory properties on ovarian cancer cell proliferation. Second, the effect of OGF on depressing the proliferation of ovarian cancer cells was eliminated by concomitant exposure to the short-acting opioid antagonist naloxone, demonstrating that OGF action was mediated by an opioid receptor. Third, using siRNA technology, knockdown of OGFr stimulated cell proliferation and neutralized the repercussions of exogenous OGF exposure, suggesting that the effects of endogenous and exogenous OGF are dependent on this non-classical opioid receptor. Fourth, administration of NTX is known to upregulate both OGF and OGFr. Fifth, continuous exposure of ovarian cancer cells to NTX accelerated cell proliferative events, suggesting that the effect of OGF not only utilized an inhibitory pathway but was tonically active in maintaining the homeostatic balance of cell replicative events. Sixth, short term opioid antagonism (i.e., LDN) initially elevates DNA synthesis during the period of opioid receptor blockade, and depresses DNA synthesis when NTX is no longer present. In view of previous evidence showing that NTX targets cell proliferative pathways, the regimen of a short term exposure to this opioid antagonist appears to have led to an enhanced interaction of the upregulated OGF and OGFr in the interval when NTX was no longer present. Thus, short term NTX mediated modulation of the OGF-OGFr axis appears to account for the depressed DNA synthesis and proliferation of human ovarian cancer cells under *in vitro* conditions. Moreover, LDN's antitumor effect in established xenografts of ovarian cancer was shown to upregulate the expression of OGF and OGFr. Therefore, we would conjecture that this upregulation of the OGF-OGFr axis by LDN intensifies the interaction of OGF and OGFr when NTX is no longer present. The net effect of LDN, therefore, is to have an exaggerated inhibitory influence on the progression of ovarian cancer. In view of these arguments, we postulate that the effects of a short-term exposure to NTX in tissue culture, and the influence of LDN on tumor progression in mice, have a common denominator in terms of mechanism: the OGF-OGFr axis.
With opioid receptor antagonism serving as a means to manipulate the opioid system related to growth, the OGF-OGFr axis, this study demonstrates that endogenous opioids are determinants of carcinogenesis and concur with previous reports. These findings raise the exciting potential in the clinical setting of utilizing LDN biotherapy alone, or in combination with chemotherapy, as novel treatment modalities for ovarian cancer. LDN has been documented to be safe for administration in humans with Crohn’s disease or multiple sclerosis. LDN has not been tested in cancer patients for safety or efficacy. However, these reports documenting the lack of toxicity of LDN in humans, and our preclinical findings that LDN is efficacious in suppressing ovarian cancer in vivo, makes the transition from the laboratory to the clinic in terms of using LDN alone or in combination with standard of care drugs feasible for the treatment of ovarian cancer. Should LDN prove to be an effective treatment for ovarian cancer, there are a number of advantages this agent would have over standard of care chemotherapies. LDN is orally effective, inexpensive, and not associated with toxic side effects. Our results suggest that LDN could be used under three different circumstances: i) as a prophylactic agent, particularly in patients with a family history of ovarian cancer, ii) as a first line treatment, alone or in combination with standard of care drugs, following cytoreductive surgery, and iii) following relapse when all other treatments are palliative.
Figures and Legends

**Figure 5.1.** The combination of short term NTX treatment with taxol or cisplatin provides an additive inhibitory effect on ovarian cancer cell number *in vitro*. (A, B) Growth curves of SKOV-3 cells subjected to (A) short term NTX (10^{-5} M, 6 h every other day) and/or taxol (10^{-9} or 10^{-10} M), continuous NTX (10^{-5} M), or an equivalent volume of sterile water, or (B) short term NTX and/or cisplatin (0.01 or 0.001 µg/mL), continuous NTX or an equivalent volume of sterile water over a 120 h period. Media and compounds were replaced daily unless otherwise noted. (C, D) Growth curves of SKOV-3 cells in reversibility experiments treated with short term NTX, continuous NTX, taxol (10^{-9} or 10^{-10} M), or cisplatin (0.01 or 0.001 µg/mL). At 48 h, a subset of cultures had media replaced without drugs (Reversal). Compounds and media were replaced daily unless otherwise indicated. (E, F) Cell number at 120 h in cultures from the reversibility experiments. Data represent cell counts (means ± SE) for at least 2 aliquots/well and 2 wells/treatment/timepoint. Significantly different from sterile water treated groups at *p*<0.05 and ***p*<0.001, from short term NTX treatment alone at ^p*<0.05, ^^p*<0.01, and ^^^p*<0.001, and from taxol or cisplatin at +p*<0.05, ++p*<0.01, and +++p*<0.001. NS = not significant.
Figure 5.2. Mechanism of enhanced growth inhibition by the combination of short term NTX treatment with taxol or cisplatin. (A, B) Evaluation of DNA synthesis by BrdU labeling in SKOV-3 cells treated with short term NTX (10^{-5} M, 6 h every other day), continuous NTX (10^{-5} M), taxol (10^{-9} or 10^{-10} M), cisplatin (0.01 or 0.001 μg/mL), short term NTX in combination with taxol or cisplatin, or an equivalent volume of sterile water (Co) for 120 h. Media and compounds were replaced daily unless otherwise indicated. Cells were pulsed with BrdU (30 μM) for 3 h prior to fixation and processed for BrdU immunoreactivity. Values represent the percent of cells labeled with BrdU (means ± SE) from at least 500 cells on 10 fields/coverglass and 2 coverglasses/treatment group. Significantly different from Co at ***p<0.001, from short term NTX treated cells at ^^p<0.01 and ^^^p<0.001, and from taxol or cisplatin alone at ++p<0.01 and +++p<0.001.
Figure 5.3. Growth of s.c. xenografts with SKOV-3 cells in mice treated with LDN, taxol, cisplatin, or LDN in combination with taxol or cisplatin chemotherapy. When tumors became visible (day 0), animals were injected with either LDN (0.1 mg/kg, daily), taxol (3 mg/kg, days 0, 7, 14, 21, 28, 35), cisplatin (4 mg/kg, days 0, 7), LDN and taxol, LDN and cisplatin, or an equivalent volume of saline (daily). (A, B) Tumor volumes were assessed 3 times/week. Data for the saline and LDN groups in A and B are from the same groups of mice. (C) Representative images of tumors before and after removal from animals following 35 days of treatment. (D) Terminal tumor volume (mm$^3$). (E) Terminal tumor weight (g). Values represent means ± SE for 10 mice/group. Significantly different from saline at *p<0.05, **p<0.01, and ***p<0.001, from LDN at ^p<0.05 and ^^p<0.01, and from cisplatin at +p<0.05, ++p<0.01, and +++p<0.001.
Figure 5.4. Body weights of mice with s.c. xenografts of ovarian cancer cells treated with LDN, taxol, cisplatin, or LDN in combination with taxol or cisplatin, with treatments initiated when tumors were visible. Values represent means ± SE body weight for 10 mice/group. The cisplatin group was significantly different from mice subjected to saline at *p<0.05 on days 2, 9, and 11, and animals receiving both LDN and cisplatin were significantly different from saline at *p<0.05 on day 2.
Figure 5.5. Mechanism of tumor growth inhibition by treatment with LDN, taxol, and/or cisplatin: effects on apoptosis, DNA synthesis, and angiogenesis. Treatments were initiated when tumors were visible (day 0) and tumor tissue was assessed 35 days later. (A) Number of apoptotic cells per 0.003 mm$^2$, as measured by the TUNEL assay. (B) % of cells with BrdU labeling. (C) Number of blood vessels per 0.16 mm$^2$, as assessed by hematoxylin/eosin staining to identify endothelial cell-lined blood vessels. Values represent means $\pm$ SE determined from at least 10 random fields from the periphery of 2 tumor sections/mouse and 2 mice/group. Significantly different from saline at $^{***}p<0.001$, from LDN at $^{*}p<0.05$ or $^{^^^}p<0.001$, and from cisplatin by $^{+}p<0.05$ or $^{++}p<0.01$. 
A

# Apoptotic Cells / 0.003 mm²

Saline  LDN  Taxol  Taxol + LDN  Cisplatin  Cisplatin + LDN

B

% BrdU Labeling

Saline  LDN  Taxol  Taxol + LDN  Cisplatin  Cisplatin + LDN

C

# Vessels / 0.16 mm²

Saline  LDN  Taxol  Taxol + LDN  Cisplatin  Cisplatin + LDN
Figure 5.6. The distribution and expression of OGF and OGFr in xenografts of SKOV-3 cells. Mice were treated with LDN, taxol, cisplatin, or LDN in combination with taxol or cisplatin beginning when tumors were visible. (A, C) Photomicrographs taken at the same exposure time of tumors on the day of sacrifice (day 35). Sections were stained with antibodies (1:200) to OGF (A) or OGFr (C). Rhodamine conjugated IgG (1:1000) served as the secondary antibody and nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (insets). Bar = 10 μm. (B, D) Semiquantitative measurement of OGF (B) and OGFr (D) staining intensity (mean gray value) from at least 10 fields from 2 sections/tumor and 3 mice/group. (E, F) Western blot of the 62 kDa band of OGFr (E) and densitometric analysis (F) normalized to β-actin from 2 independent experiments. (G) Saturation isotherms calculating the binding capacity (B<sub>max</sub>) of OGFr in xenografts from at least 3 independent assays performed in duplicate. Data represent means ± SE. Significantly different from saline at *p<0.05, **p<0.01 and ***p<0.001.
A

B

C

D

E

F

G

OGF

DAPI

Merged

OGFr

DAPI

Merged

OGFr

Actin

Saline + + + - - -
LDN - - + + +

Binding Capacity (nmol/mg protein)

Saline LDN Taxol LDN + Taxol LDN + Cisplatin Cisplatin + LDN

% of Control

0 50 100 150 200 250

Saline LDN

*** ** ** **

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References


Chapter 6: OGF and LDN Treatments in Intraperitoneal Xenograft Model

Rationale
Modulation of the OGF-OGFr axis by treatment with OGF or LDN has been shown to inhibit tumor progression in mice with established subcutaneous xenografts. However, the role of the OGF and LDN on tumor growth of cells implanted and grown in the intraperitoneal cavity of mice needs to be investigated. This clinically relevant study allows a direct comparison of the effects of OGF and LDN, as these treatments were administered in the same study.
The Opioid Growth Factor (OGF) and Low Dose Naltrexone (LDN) Suppress Human Ovarian Cancer Progression in Mice

Accepted for publication in Gynecologic Oncology

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Abstract

Objective: The opioid growth factor (OGF) and its receptor, OGFr, serve as a tonically active inhibitory axis regulating cell proliferation in normal cells and a variety of cancers, including human ovarian cancer. Blockade of OGF and OGFr with the nonselective opioid receptor antagonist naltrexone (NTX) upregulates expression of OGF and OGFr. Administration of a low dosage of NTX (LDN) blocks endogenous opioids from opioid receptors for a short period of time (4–6 h) each day, providing a window of 18–20 h for the upregulated opioids and receptors to interact. The present study investigated the repercussions of upregulating the OGF-OGFr axis by treatment with OGF or LDN on human ovarian tumorigenesis in vivo.

Methods: Female nude mice were transplanted intraperitoneally with SKOV-3 human ovarian cancer cells and treated on a daily basis with OGF (10 mg/kg), LDN (0.1 mg/kg), or an equivalent volume of vehicle (saline). Tumor burden, as well as DNA synthesis, apoptosis, and angiogenesis were assessed in tumor tissue following 40 days of treatment.

Results: OGF and LDN markedly reduced ovarian tumor burden (tumor nodule number and weight). The mechanism of action was targeted to inhibition of tumor cell proliferation and angiogenesis; no changes in cell survival were noted.

Conclusions: This study shows that a native opioid pathway can suppress human ovarian cancer in a xenograft model, and provides novel non-toxic therapies for the treatment of this lethal neoplasia.
Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies [1], and is the 5th leading cause of cancer mortality among women in the United States [2]. Approximately 75% of these women present in the advanced stages. Although clinical response to cytoreductive surgery and adjuvant chemotherapy is excellent [3], 65% of patients relapse within 2 years and thereafter only receive palliative care [1]. An understanding of the pathogenesis of ovarian cancer will be required in order to exploit biological pathways for treatment [1].

Dysregulation of cell proliferation is a fundamental component of the ovarian cancer phenotype [4]. The opioid growth factor (OGF) and its receptor (OGFr) have been reported to be a native biological regulator of cell replication in human ovarian cancer cells using a tissue culture model [5]. OGF, chemically termed [Met$^5$]-enkephalin, is a constitutively active native opioid peptide that interacts with OGFr to upregulate cyclin-dependent kinase inhibitory (CKI) pathways and markedly delay the G$_1$/S phase of the cell cycle [5-8].

This study examined whether OGF depresses human ovarian cancer in vivo using an intraperitoneal xenograft model with parallels to the human situation. In addition, we have investigated another means to modulate the OGF-OGFr axis to alter the course of ovarian carcinogenesis using a low dose of the opioid antagonist naltrexone (LDN). LDN blocks endogenous opioids from opioid receptors for a short period of time (4–6 h), producing an upregulation of opioid systems [9-11]. For the remaining 18-20 h window each day, the elevated opioids and receptors interact to elicit a robust functional effect (e.g., growth inhibition) [10, 11]. We now show that both OGF and LDN have a marked effect on suppressing the progression of human ovarian cancer, suggesting that these agents may warrant clinical consideration as treatment modalities.
Material and methods

Cell culture

The human epithelial ovarian cancer cell line SKOV-3 [12], obtained from the American Type Culture Collection (Manassas, VA), was grown in a humidified atmosphere of 5% CO$_2$/95% air at 37°C in RPMI medium supplemented with 1.2% sodium bicarbonate, 10% fetal calf serum, 5,000 units/ml penicillin, 5 μg/ml streptomycin, and 10 mg/ml neomycin.

Animals, xenografts, and treatments

Four week-old athymic nu/nu female mice, purchased from Charles River Laboratory (Wilmington, MA), were housed in pathogen-free isolator-ventilated cages in a controlled-temperature room (22-25°C) with a 12-12 h light/dark cycle (lights on 0700-1900). Sterile rodent diet (Teklad, Indianapolis, IN) and water were available ad libitum. All procedures were approved by the IACUC committee of Penn State University College of Medicine, and conformed to the guidelines established by the NIH. Based on published reports [13, 14], as well as preliminary tumor burden studies, unanaesthetized mice were injected intraperitoneally with SKOV-3 cells (5 x 10$^6$/mouse) following a 48 h acclimation period.

Within 1 h of tumor cell inoculation, three groups of mice were randomized to receive daily intraperitoneal injections of OGF (10 mg/kg, n=8), LDN (0.1 mg/kg, n=8), or an equivalent volume (0.2 ml) of saline (n = 16); dosages were selected based on published reports [15-18]. LDN and OGF were obtained from Sigma Aldrich (St. Louis, MO), dissolved in saline, and prepared on a weekly basis. The abdomens of mice were examined for distension and mice were weighed 3 times/week.

Termination day measurements

Following 40 days of treatment, mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) followed by cervical dislocation. For examination of DNA synthesis in tumors, a subset of mice was injected intraperitoneally with 100 mg/kg BrdU at 6 and 3 h prior to euthanasia. The number of tumor nodules on the surfaces of the liver, stomach, spleen, and mesentery/intestines were recorded at necropsy, and the cumulative tumor weight for each mouse determined. Tumors were processed for
semiquantitative immunohistochemistry, as well as receptor binding, TUNEL, hematoxylin/eosin, and BrdU analysis.

**Semiquantitative immunohistochemistry**

Semiquantitative immunohistochemistry was utilized to evaluate the presence and relative level of OGF and OGFr in tumor tissue [5, 19, 20]. Tumors were excised, frozen in chilled isopentane, sectioned at 10 μm, and stained with antibodies to OGF and OGFr generated in our laboratory [21]. To evaluate expression, images were taken at the same exposure time. At least 10 fields/section from 2 sections/tumor and 3 tumors/group were utilized. Controls were incubated with secondary antibodies only.

**OGFr binding assays**

Tumors were assayed for OGFr binding using custom synthesized [³H]-[Met⁵]-enkephalin (Perkin Elmer, Waltham, MA; 52.7 Ci/mmol) following published procedures [5, 19, 20]. Saturation binding isotherms were generated using GraphPad Prism software (La Jolla, CA), and independent assays were performed at least 2 times.

**Mechanism of tumor inhibition: Apoptosis, DNA synthesis, and angiogenesis**

The effects of treatments on DNA synthesis (BrdU incorporation), apoptosis (TUNEL), and vasculature (hematoxylin/eosin staining to identify endothelial lined vessels containing red blood cells) were evaluated. Tumors from mice receiving BrdU were fixed overnight in formalin, processed in paraffin, and sectioned at 10 μm. Tissue was stained with antibody to BrdU (1:200, Invitrogen) [19, 20], hematoxylin/eosin [22-25], or processed for TUNEL (Trevigen, Gaithersburg, MD). The percent of BrdU positive cells, number of TUNEL positive cells, and blood vessel density were determined from at least 10 random fields around the periphery of 2 sections/tumor, and 2 tumors/treatment group.

**Statistical analysis**

All data was analyzed using one way analysis of variance (ANOVA) with subsequent comparisons made using Newman–Keuls tests (Graph Pad Prism Software).
Results

Body weight and gross observations

Body weights of mice administered OGF, LDN, or saline and inoculated with SKOV-3 cancer cells were comparable throughout the 40-day study (data not shown). Similarly, at the time of euthanasia, no changes in terminal spleen weight were noted in mice injected with tumor cells and receiving OGF, LDN, or saline, and no behavioral or ingestive (e.g., fluid consumption, feeding) abnormalities were noted in any group (data not shown).

OGF and LDN inhibit tumor burden in mice with intraperitoneal xenografts

Following 40 days of treatment, 100% of mice receiving saline or LDN treatments, and 87% of mice receiving daily injections of OGF, developed macroscopically visible tumors within the peritoneal cavity. Tumor nodules were detected predominantly on the surface of the liver, stomach, spleen, and intestines, as well as on the walls of the body cavity (Figure 6.1A). OGF and LDN treated mice displayed a visible reduction in tumor burden relative to saline administered controls. No apparent metastases were located beyond the peritoneal cavity (e.g., lungs, heart), and ascites was not observed in any group. Compared to the total number of nodules detected in mice administered saline (39.4 ± 2.8), animals treated with OGF or LDN displayed a 42% and 39% reduction, respectively (Figure 6.1B). In contrast to saline-injected animals, mice receiving OGF and LDN had a 65% and 51% reduction, respectively, in the number of nodules on the liver, a 69%, and 27% diminution, respectively, in the quantity of lesions on the stomach, and a 33% and 38% decrease, respectively, in the number of tumors on the intestines/mesentery (Figure 6.1B). With regard to tumor nodules on the spleen, comparable numbers were noted in all groups (Figure 6.1B). Relative to the cumulative tumor weight recorded in saline controls, tumor weight was reduced in mice treated with OGF (69%) and LDN (46%) (Figure 6.1C).

The presence and expression of OGF and OGFr in xenografts of mice injected with ovarian cancer cells

To evaluate OGF and OGFr distribution and expression, semiquantitative immunohistochemistry and receptor binding were performed on tumors from mice
treated with OGF, LDN, or saline. The location of OGF was similar in tumor tissue from all groups, with immunoreactivity detected in the cytoplasm, and a speckling often noted in cell nuclei (Figure 6.2A). OGF immunofluorescence (mean gray value) was increased 57% and 47% in tumors from mice treated with OGF or LDN, respectively, relative to those of saline controls (Figure 6.2B). The distribution of OGFr immunoreactivity in tumor tissue was detected in the cytoplasm and often lightly scattered in cell nuclei (Figure 6.3A). Relative to saline controls, OGFr expression as assessed by semiquantitative immunohistochemistry was decreased 29% in tumors from mice treated with OGF, and increased 63% in tumors from animals administered LDN (Figure 6.3B). Tumors processed with only secondary antibody showed no staining (Figures 6.2A, 6.3A inset).

Utilizing receptor assays, specific and saturable binding to OGF was detected in the nuclear fraction of tumor tissue, with a one site model of binding recorded in all groups (Figure 6.3C). Binding capacity (B_max) was reduced in tumor tissue from mice receiving OGF (61%), but increased (90%) in animals administered LDN, compared to preparations from saline injected control mice (Figure 6.3C). Binding affinity (K_d) ranged from 4.7 to 9.8 nM, and did not differ in tumors from mice receiving daily injections of OGF, LDN, or saline (data not shown).

**Mechanism of tumor growth inhibition: Apoptosis, DNA synthesis, and angiogenesis**

Examination of apoptosis by the TUNEL assay revealed comparable levels of programmed cell death (1.6-1.9 cells/0.003 mm²) in tumors from mice treated with OGF, LDN or saline (Figures 6.4A, B). In contrast, relative to BrdU labeling rates in tumors from animals receiving saline (23.1± 2.3%), BrdU labeling was reduced 61% and 52% in tumors from mice administered OGF or LDN, respectively (Figures 6.4C, D). Similarly, blood vessel density in tumors was decreased in mice receiving OGF (89%) or LDN (73%), compared to saline administered controls (Figures 6.4E, F).
Discussion

The present study demonstrates for the first time that upregulation of the OGF-OGFr axis, by daily treatment with OGF or LDN, has a potent inhibitory effect on human ovarian tumorigenesis in a clinically relevant intraperitoneal xenograft model [13, 26]. Exposure to OGF or LDN significantly reduced the number of macroscopic tumor nodules in the peritoneal cavity of mice, as well as inhibited the cumulative weight of these neoplasms. The mechanism of inhibition by OGF and LDN treatments was not associated with an induction of apoptosis, but instead was associated with an inhibitory effect on DNA synthesis and angiogenesis in tumor tissue. These studies imply that targeting a native biological pathway, the OGF-OGFr axis, can markedly alter the course of a deadly human cancer.

Toxicity with chemotherapeutic agents has been a major drawback accompanying treatment of ovarian cancer. Importantly, in the present study no systemic toxicity was noted in mice receiving OGF or LDN, with comparable body weights and behavior noted in these groups as recorded in the saline control group. This lack of toxicity by modulating agents of the OGF-OGFr axis in the present study is consonant with previous reports on other cancers (e.g., pancreatic, squamous cell carcinoma of the head and neck) [11, 16-18, 27, 28]. Thus, not only can OGF or LDN suppress the progression of ovarian cancer, but they do so in the absence of toxic side-effects.

A number of lines of evidence indicate that, although naltrexone is a non-selective opioid receptor antagonist, the endogenous opioid peptide-receptor system upregulated by LDN during the period of opioid receptor blockade to inhibit ovarian cancer cell proliferation and tumorigenesis is the OGF-OGFr axis. First, amongst a panel of natural and synthetic opioids, many specific for classic opioid receptors (\(\mu, \delta, \kappa\), OGF was the singular opioid peptide with growth inhibitory properties on ovarian cancer proliferation \textit{in vitro} [5]. Second, using siRNA technology to knockdown OGFr in human ovarian cancer cells, this receptor was demonstrated to be specific in mediating the inhibitory actions of OGF [5, 29]. Third, previous studies, as well as the findings in the current report, demonstrate that naltrexone upregulates both OGF and OGFr [30, 31]. Finally, the fact that LDN was found to inhibit cell proliferation without inducing
apoptosis supports mediation through an opioid peptide-opioid receptor system that inhibits cell proliferation without affecting cell survival. This is in contrast to the induction of apoptosis reported with an interaction of opioids with classic opioid receptors [32], and is consistent with the interfacing of OGF and OGFr, which inhibit cell proliferation by targeting the G1/S phase of the cell cycle without inducing apoptosis [5-8, 33].

The evidence in the present study reveals that upregulation of OGF-OGFr by exogenous OGF or LDN inhibits tumorigenesis through a reduction in cell proliferation and angiogenesis, but not an induction of apoptosis. These data on the OGF-OGFr axis targeting cell proliferative and angiogenic pathways extend observations in previous reports [11, 18, 34-38]. With respect to ovarian cancer, in tissue culture OGF has been shown to inhibit cell proliferation by upregulating the p16/p21 CKI pathways, which in turn stalls cells at the G1/S phase of the cell cycle, but does not affect cell survival [5]. Furthermore, both OGF and LDN have been shown to reduce cell proliferation in mice with neuroblastoma xenografts [36]. In that study, DNA synthesis was initially reported to be increased during the period of opioid receptor blockade (4-6 h) by LDN, but was markedly depressed in the subsequent 18-20 h period when naltrexone was no longer present, resulting in a net inhibition on cell proliferation and tumorigenesis [36]. Also consonant with the present findings, OGF has been shown to inhibit the number of vessels and total vessel length in a chick chorioallantoic membrane model system [37, 38]. Thus, we have found that the progression of ovarian cancer can be shaped by two different means, exogenous OGF and LDN, both of which are directed to modulating a common denominator: the OGF-OGFr axis. However, it should be noted that LDN upregulated both OGF and OGFr for a gain in function. In contrast, increasing the magnitude of the OGF-OGFr axis by way of exogenous OGF, resulted in a downregulation of OGFr, presumably because the overwhelming concentration of exogenous OGF could sufficiently meet the demand of OGFr needed to depress cell proliferation.

Although most women with ovarian cancer initially respond to cytoreductive surgery and chemotherapy, recurrence of carcinogenesis often occurs, with only palliative therapies available [39]. The peptide, OGF, utilized in the present study has
been documented as safe for administration in humans [40], and has been shown to have efficacy in phase II trials of patients with advanced pancreatic cancer [41]. LDN also has been tested in the clinic and found to be safe and efficacious for humans with Crohn's disease [42]. However, LDN has not yet been evaluated in cancer patients. Should OGF and LDN prove to be effective therapies for ovarian cancer, they would have a number of advantages over current chemotherapeutic approaches. For example, LDN is not toxic, orally effective, and inexpensive [43]. The evidence presented herein demonstrate that the OGF-OGFr axis is a determinant of ovarian cancer progression in a clinically relevant model of human ovarian cancer, and suggest that treatment with OGF or LDN merits consideration as a therapy for ovarian cancer.
**Figure 6.1.** Terminal tumor measurements at 40 days in mice with SKOV-3 intraperitoneal xenografts treated with OGF (10 mg/kg), LDN (0.1 mg/kg), or 0.2 ml of saline. (A) Representative images of the peritoneal cavity; arrows indicate tumors. (B) Number of tumor nodules. (C) Total tumor weight. Data represent means ± SE. Significantly different from the saline group (Co) at *p<0.05, **p<0.01, and ***p<0.001.
Figure 6.2. The distribution and expression of OGF in tumors from mice with SKOV-3 intraperitoneal xenografts and treated with OGF, LDN, or saline for 40 days. (A) Photomicrographs of tumors stained with antibodies to OGF (1:200) were taken at the same exposure time. Rhodamine conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (inset). Arrows indicate OGF immunoreactivity. Bar = 40 μm. (B) Semiquantitative measurement of OGF immunoreactivity (mean gray value) from at least 10 fields/section, 2 sections/tumor, and 3 mice/group. Data represent means ± SE. Significantly different from the saline group (Co) at ***p<0.001.
Figure 6.3. The distribution and expression of OGFr in tumors from mice inoculated intraperitoneally with SKOV-3 cells and treated daily with OGF, LDN, or saline for 40 days. (A) Photomicrographs of tumors stained with antibodies to OGFr (1:200) were taken at the same exposure time. Rhodamine conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (inset). Arrows indicate OGFr immunoreactivity. Bar = 40 μm. (B) Semiquantitative measurement of OGFr immunoreactivity (mean gray value) from at least 10 fields/section, 2 sections/tumor, and 3 mice/group. (C) Saturation isotherms calculating the binding capacity (B$_{\text{max}}$) of OGFr in the nuclear fraction of tumor tissue for radiolabeled OGF from at least 2 independent assays performed in duplicate. Data represent means ± SE. Significantly different from the saline group (Co) at **p<0.01 and ***p<0.001.
Figure 6.4. Mechanism of tumor growth inhibition in mice with SKOV-3 intraperitoneal xenografts and treated daily with OGF, LDN, or saline for 40 days: Effects on apoptosis, DNA synthesis, and angiogenesis. (A) Photomicrographs of tumor tissue stained with TUNEL. Negative and positive controls were included according to the manufacturer’s instruction. (B) Number of apoptotic cells per 0.003 mm². (C) Photomicrographs of BrdU staining. (D) The percent of cells with BrdU labeling. (E) Photomicrographs of hematoxylin/eosin staining identifying endothelial lined vessels containing red blood cells. (F) Number of blood vessels per 0.16 mm². Arrows in A, C, and E indicate positive staining. Data in B, D, and F were determined from at least 10 fields/section, 2 sections/tumor, and 2 mice/group. Scale bar = 40 μm in A and C, and 120 μm in E. Significantly different from the saline group (Co) at ***p<0.001.
References


Chapter 7: Stable Overexpression of OGFr

In Vitro and In Vivo

Rationale
The importance of the OGF-OGFr axis in the regulation of ovarian cancer cell proliferation and tumor growth has been demonstrated thus far through studies evaluating the effects of OGF or LDN treatment, both in vitro and in vivo. LDN treatment was shown to upregulate both OGF and OGFr during the period of opioid receptor blockade, allowing an enhanced interaction of OGF and OGFr during the period when NTX is not present. However, NTX is a non selective opioid receptor antagonist known to modulate the expression of other opioid receptors. The present study was designed to evaluate the repercussions of chronically amplifying OGFr in human ovarian cancer both in vitro and in vivo by stably transfecting SKOV-3 cells to molecularly overexpress OGFr. Because the OGF-OGFr axis is a tonically active inhibitory system, it is expected that cell proliferation and tumor growth will be repressed through interaction of the upregulated OGFr with endogenous OGF. The findings of this study are expected to support treatments that amplify OGFr to decrease the growth of human ovarian cancer.
Overexpression of the opioid growth factor receptor (OGFr) in human ovarian cancer cells downregulates cell proliferation \textit{in vitro} and inhibits tumorigenesis in mice

Submitted for publication

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Abstract

**Background:** The majority of patients with ovarian cancer present in advanced stages, and eventually relapse with only palliative therapies available. The opioid growth factor (OGF) and its receptor, OGFr, serve as a tonically active inhibitory axis regulating the proliferation of human ovarian cancer cells. In the present study, we have asked the question of whether molecular overexpression of OGFr is a determinant of the progression of this deadly neoplasia.

**Results:** The human ovarian cancer cell line, SKOV-3, was transfected with an expression vector to overexpress OGFr. Five clonal cell lines were evaluated, and untransfected wild-type (WT) cells and empty vector (EV) transfected clones served as controls. Under standard growth conditions clonal cell lines overexpressing OGFr had decreased cell numbers and increased doubling times compared to WT and EV controls. Subcutaneous xenografts with cells overexpressing OGFr exhibited increases from controls in the time to form a measurable tumor, decreases in the number of animals displaying a measurable tumor, and reductions in tumor volume and weight. Mice injected by the intraperitoneal route with ovarian cancer cells containing an abundance of OGFr exhibited tumor nodules and weights that were markedly reduced from control levels. DNA synthesis, but not cell survival, was associated with the depressed growth of cells and tumors with a molecular amplification of OGFr.

**Conclusion:** Overexpression of OGFr in human ovarian cancer cells is now shown to have a profound inhibitory effect on cell proliferation and tumorigenesis. These data indicate that OGFr is a molecular determinant in the progression of human ovarian cancer, and has important relevance for the design of treatment strategies for this deadly disease.
Background

Ovarian cancer is the 5th leading cause of cancer related mortality among women in the United States, and the leading cause of death from gynecological malignancies [1]. Ninety percent of primary ovarian cancers are epithelial in origin [2]. Eighty percent of patients present in advanced stages (FIGO stage III/IV), with cytoreductive surgery and adjuvant chemotherapy serving as treatment [3]. Nearly 75-80% of women initially respond to treatments; however, approximately 65% of these patients relapse within 12 to 18 months of therapy [4]. Once ovarian cancer recurs, therapeutic modalities are only palliative [3]. The cellular and molecular events involved in ovarian cancer pathogenesis need to be defined, and major improvements in treatment will require new therapies based on exploitation of biological pathways [4].

An integral component of the ovarian cancer phenotype is dysregulation of cell proliferation [5]. One native biological regulator of cell replication in normal cells and a wide variety of cancers, including ovarian cancer, is the opioid growth factor (OGF) and its receptor, OGFr [6-11]. Chemically termed [Met^5]-enkephalin, OGF is a constitutively active native opioid peptide that is autocrine produced and secreted, and interacts with OGFr to delay the G1/S interface of the cell cycle by upregulating cyclin-dependent kinase inhibitory (CKI) pathways [11-14] without affecting cell survival [11, 15]. Although OGFr has pharmacological characteristics of classical opioid receptors (recognizes opioids, naloxone reversibility, stereospecificity), this receptor shares no homology with classical opioid receptors at the nucleotide or amino acid level, and has a different cellular localization [6, 16-19]. Regulation of cell proliferation by the OGF-OGFr axis involves nucleocytoplasmic trafficking from the outer nuclear envelope to the nucleus that requires nuclear localization signals and transport by karyopherin β and Ran [17, 20]. An increase in the OGF-OGFr pathway by the addition of exogenous OGF [7-11, 21], treatment with imidazoquinoline compounds such as imiquimod and resiquimod which upregulate OGFr [22], or transfection of sense cDNA for OGFr [23, 24], depresses cell proliferation.

The relationship of endogenous opioids and ovarian cancer has received some attention. In 1982, Sporrong et al. [25] recorded immunoreactive enkephalin in primary ovarian carcinoids, whereas Zagon et al. [26] in 1987 noted δ and κ, but not μ, opioid
receptors in an ovarian fibroma and stromal hyperplasia tumor, and detected both [Met$^5$]-enkephalin and β-endorphin in an ovarian fibroma. In 1989, Kikuchi and colleagues [27] reported that β-endorphin, α-endorphin, and [Met$^5$]-enkephalin (i) inhibited the growth of human serous cytoadenocarcinoma cells of the ovary in vitro in a dose-dependent manner that was partially reversed by the opioid antagonist, naloxone, and (ii) decreased protein and RNA synthesis but not DNA synthesis. In a subsequent study, Kikuchi et al. [28] noted that β-endorphin (but not α-endorphin or [Met$^5$]-enkephalin) increased lytic activity in spleen cells from mice bearing human ovarian carcinoma, and speculated that opioid peptides play a role in immune surveillance mechanisms. Finally, Mollick et al. [29] reported antibodies to OGFr in the serum of 4 ovarian cancer patients, and suggested that OGFr may be a "useful target for vaccination."

Donahue and collaborators [11] have made substantial progress in clarifying the interplay between ovarian cancer and opioid peptides and receptors using an in vitro model of human ovarian cancer. These investigators identified OGF, acting in the capacity of regulating cell proliferation, as the only opioid peptide involved. Moreover, OGFr was discovered to be the opioid receptor that was in an autocrine loop with OGF with respect to maintaining the pace of cell replication. Knockdown of OGFr in the ovarian cancer cells using siRNA resulted in an increase in cell number relative to vehicle controls, and exogenous OGF introduced into these cultures did not depress cell proliferation [11, 21]. In the present report we have asked the question as to the repercussions of having an upregulation of OGFr in human ovarian cancer cells. We found that stable molecular overexpression of OGFr in vitro decreased cell proliferation, and under in vivo conditions using xenografts with either the subcutaneous or intraperitoneal routes increased the latency to the development of ovarian tumors and markedly decreased tumor volume, number, and weight. Moreover, the increase in OGFr that attenuated tumorigenesis was related to a reduction in DNA synthesis but not to alteration in cell survival. These results demonstrate the critical nature of the OGF-OGFr axis in the onset and progression of human ovarian cancer, and will be important in the design of treatment strategies for this deadly disease.
Methods

Cell culture

The human ovarian cancer cell line, SKOV-3 [30], was obtained from the American Type Culture Collection (Manassas, VA), and grown in a humidified atmosphere of 5% CO$_2$/95% air at 37$^\circ$C in RPMI medium containing 1.5 mM L-glutamine, 2.2 g/l sodium bicarbonate, 10% fetal calf serum, and antibiotics (5,000 units/ml penicillin, 5 µg/ml streptomycin, and 10 mg/ml neomycin).

Transfection and clonal selection

SKOV-3 cells were transfected with pcDNA3.1+ vector (empty vector, EV) or with the plasmid pcDNA3.1+ human OGFr in the presence of lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 4 hours in serum and antibiotic free media. At 4 h, cultures were supplemented with serum containing media. At 24 h, transfection reagents were removed and replaced with serum containing media. Transfected cells were selected by growth in media containing G418 at 500 µg/ml; 24 clones were expanded and analyzed by Western blotting. Based on OGFr expression, 5 clones (OGFr-3, OGFr-13, OGFr-15, OGFr-21, and OGFr-22) were maintained and further characterized by semiquantitative immunohistochemistry, Western blot, receptor binding assays, and growth. For all experiments, untransfected wild-type (WT) cells and EV transfected clones served as controls.

Cell growth

Clonal cells, as well as WT and EV cells, were plated and counted 24 hours later (time 0) to determine seeding efficiency. For treatment studies, $10^{-6}$ M OGF or naltrexone (NTX) (Sigma Aldrich, St. Louis, MO) was added at time 0; media and compounds were replaced daily. Drugs were prepared in sterile water and dilutions represent final concentrations of the compounds. An equivalent volume of sterile water was added to controls. At designated times, cells were harvested, stained with trypan blue, and counted with a hemacytometer. At least two aliquots/well from at least 2 wells/treatment/timepoint were sampled.

Animals

Four week-old athymic nu/nu female mice, purchased from Charles River Laboratory (Wilmington, MA), were housed in pathogen-free isolator ventilated cages in...
a controlled-temperature room (22-25°C) with a 12-12 hour light/dark cycle (lights on 0700-1900) in the Department of Comparative Medicine at The Pennsylvania State University College of Medicine. Sterile water and standard rodent diet (Teklad, Indianapolis, IN) were available ad libitum. All procedures were approved by the IACUC committee of The Pennsylvania State University College of Medicine, and conformed to the guidelines established by the NIH. Mice were allowed 48 hours to acclimate prior to experimentation.

**Tumor cell implantation**

Clonal cell lines stably overexpressing OGFr, OGFr-3, and OGFr-22, as well as EV and WT cells, were expanded and analyzed by receptor binding to determine the binding capacity of OGFr prior to inoculation into mice. For the subcutaneous xenograft model, 4 x 10^6 WT, EV, OGFr-3, or OGFr-22 cells were injected into the right scapula region (~0.1 ml/mouse) of unanaesthetized mice. For the intraperitoneal xenograft model, unanaesthetized mice were injected with 5 x 10^6 EV, OGFr-3, or OGFr-22 cells. These concentrations were selected based on published reports [31-34] as well as preliminary tumor burden studies (Donahue et al., unpublished observations).

**Tumor growth and termination day measurements**

Mice with subcutaneous xenografts were weighed weekly and observed daily for initial appearance of a visible tumor. The latency for a visible tumor and the time until tumors were measurable (≥62.5 mm^3) were recorded. Tumors were measured in two dimensions with vernier calipers 3 times/week. Volume was calculated using the formula l x w^2 x π/6, where length (l) is the longest dimension, and width (w) is the dimension perpendicular to the length [35]. Mice with intraperitoneal xenografts were weighed 3 times/week and their abdomens examined for distension throughout the study.

Mice were euthanized 32 and 40 days following subcutaneous or intraperitoneal tumor cell inoculation, respectively, by an overdose of sodium pentobarbital (100 mg/kg) and cervical dislocation. To examine DNA synthesis in tumors, a subset of mice from each group was injected intraperitoneally with BrdU (100 mg/kg) at 6 and 3 hours prior to euthanasia. Mice with subcutaneous xenografts were weighed, tumors and spleens were removed and weighed, and the lymph nodes, liver, and spleen were examined for
metastases. Tumors were processed for immunohistochemistry, BrdU, hematoxylin and eosin, and TUNEL analysis. Mice with intraperitoneal xenografts were weighed and the number of tumor nodules on the surfaces of the liver, stomach, spleen, and intestines were recorded, removed, and weighed.

**Semiquantitative immunohistochemistry**

Semiquantitative immunohistochemistry was utilized to evaluate the presence and relative levels of OGF and OGFr in cells and tumor tissue. Log phase cells were plated onto 22 mm round coverglasses, and grown in culture for 72 h, while tumors were excised, frozen in isopentane chilled on dry ice at the time of euthanasia, and sectioned at 10 μm. Cells and tissues were fixed and permeabilized with ice cold ethanol (95% vol/vol) and acetone (100%), and immediately stained with anti-OGF or anti-OGFr antibodies according to published procedures [11]. Polyclonal antibodies to OGF and OGFr were generated in the laboratory and have been fully characterized [36]. To evaluate the relative expression of OGF and OGFr, images were taken at the same exposure time with care not to photobleach samples. For cells, the mean intensity of staining (mean gray value) was determined for at least 100 cells/group, and at least 3 coverglasses/group. For tumors, at least 10 fields/section from the periphery of 2 sections/tumor, and 3 tumors/group were assessed. Controls included preparations incubated with secondary antibodies only.

**Protein isolation and Western blotting**

Expression of OGFr was evaluated in clonal lines by Western blot according to published procedures [11]. Briefly, cells were harvested, solubilized in RIPA buffer (PBS, 10 μM IGEPAL, 1 mg/ml SDS) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN), sheared with a 25 G needle, and total protein concentrations measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins (60 μg) were subjected to 15% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with anti-OGFr (1:200) or β-actin (1:5000, Sigma Aldrich) antibodies followed by appropriate secondary horseradish peroxidase conjugated antibodies (1:5000, GE Healthcare-Amersham Biosciences, Piscataway, NJ). The optical density of each band was determined by densitometry (QuickOne, Bio-Rad Laboratories), and each value was normalized to β-actin from the same blot. The
percent change in expression was calculated by dividing the normalized value of experimental samples by that of WT samples. Means and SE were determined from at least 2 independent experiments.

**OGFr binding assays**

Log phase cells were assayed for OGFr using custom synthesized $[^3]$H-$[\text{Met}^5]$-enkephalin (Perkin Elmer- New England Nuclear; 52.7 Ci/mmol) following procedures by Donahue et al [11]. Non-specific binding was measured in the presence of unlabeled $[\text{Met}^5]$-enkephalin. Saturation binding isotherms were generated using GraphPad Prism (La Jolla, CA), and independent assays were performed in duplicate at least 3 times.

**Mechanism of modulated growth: DNA synthesis, apoptosis, and necrosis**

The effect of overexpressing OGFr on DNA synthesis, apoptosis, and necrosis was assessed in cells. Tumor tissue was evaluated for DNA synthesis and apoptosis. Cells ($5 \times 10^4$/coverglass) were grown in culture for 72 h, pulsed with BrdU ($30 \mu M$, Sigma Aldrich) for 3 h, and fixed in formalin. Tumors from mice receiving BrdU were fixed in formalin overnight, processed in paraffin, and sectioned at 10 μm. Cells or tissue was stained with anti-BrdU antibody (1:200, Invitrogen) to assess DNA synthesis [11, 23, 24], or processed for TUNEL according to the manufacturer’s instruction (Trevigen, Gaithersburg, MD) to measure apoptosis [11]. For cells, the percentage of BrdU or TUNEL positive cells was determined for at least 500 cells/group and at least 2 coverglasses/group. For tumors, the percentage and number of BrdU and TUNEL positive cells, respectively, were determined from at least 10 random fields around the periphery of each tumor, with 2 sections/tumor, and 2 tumors/treatment group evaluated. Necrosis was assessed in cells by trypan blue staining in all growth experiments.

**Histological staining**

Staining with hematoxylin and eosin was performed on tumor tissue to examine endothelial lined vessels containing red blood cells [37, 38]. Blood vessel density was determined from at least 10 random fields around the periphery of each tumor, with 2 sections/tumor, and 2 tumors/treatment group evaluated.
Statistical analysis

Tumor incidence was analyzed using the Chi square test; all other data was analyzed using one-way analysis of variance (ANOVA) with subsequent comparisons made using Newman-Keuls tests (GraphPad Prism). In some cases, data were evaluated using unpaired t-tests. P values <0.05 were considered to be significant.
Results

Establishment and characterization of OGFr clones

To study the effects of amplification of OGFr on the growth of ovarian cancer cells, SKOV-3 cells were stably transfected with an OGFr expression vector; 24 neomycin-resistant clones were initially characterized by Western blot (data not shown). Five clones with varying levels of OGFr expression as compared to the WT and EV cultures were expanded and further characterized by semiquantitative immunohistochemistry, Western blot, OGFr receptor binding, and growth (Figures 7.1, 7.2). For all studies, comparisons were made to WT and EV groups.

OGFr is present and overexpressed in clones

For all cultures, OGFr was visible in the cytoplasm and a speckling of immunoreactivity often noted in cell nuclei (Figure 7.1A). Cells processed with secondary antibody only showed no staining (Figure 7.1A inset). Semiquantitative immunohistochemistry revealed a marked increase in OGFr immunofluorescence (mean gray value) in clonal cell lines OGFr-3 (213%), OGFr-13 (158%), OGFr-15 (62%), OGFr-21 (245%) and OGFr-22 (87%) relative to WT cells (Figure 7.1B). Western blot analysis revealed that the 62-kDa band of OGFr, standardized for loading with actin, was increased 77-117% in clones overexpressing OGFr relative to WT cells (Figure 7.1C). No differences were noted between WT and EV cells by Western blotting or semiquantitative immunohistochemistry.

Overexpression of OGFr upregulates receptor binding capacity

To further characterize OGFr overexpressing clones, binding capacity ($B_{\text{max}}$) and binding affinity ($K_d$) of OGFr for radiolabeled [Met$^5$]-enkephalin was determined. Specific and saturable binding was identified in the nuclear fraction of all cell lines. $B_{\text{max}}$ values for clonal cells were markedly increased compared to those of WT (3.84 ± 0.21) and EV (3.45 ± 0.29) groups, with increases ranging from 51-154% in cells with amplified OGFr (Figure 7.1D). Binding affinity did not differ between WT, EV, and clonal cell lines (data not shown).

Overexpression of OGFr downregulates cancer cell proliferation

The functional repercussions of overexpressing OGFr were determined by evaluating cell growth. Cell number was significantly decreased 36-85% over a 120
hour period in clonal lines overexpressing OGFr compared to EV and WT controls (Figure 7.2A). Calculation of doubling times (Figure 7.2B) revealed significant increases in OGFr-3 (121%), OGFr-13 (48%), OGFr-15 (41%), OGFr-21 (80%), and OGFr-22 (177%) cell lines relative to WT and EV control levels. Doubling times between WT and EV groups were comparable (~ 34 h).

**Exogenous OGF decreases cell number in cultures overexpressing OGFr**

In earlier studies the addition of exogenous OGF to ovarian cancer cultures depressed cell number [11, 21], leading to the prediction that exogenous OGF introduced to cells with amplification of OGFr would result in an exaggerated inhibitory response. Under standard growth conditions, the basal number of cells in cultures overexpressing OGFr was reduced 70-85% compared to WT and EV cultures (Figure 7.2C). The addition of exogenous OGF (10^-6 M) to clonal cell lines depressed cell number an additional 19-34% compared to their respective basal levels at 120 hours (Figure 7.2C). Summation of the basal growth inhibition plus growth inhibition induced by exogenous OGF at 120 hours revealed a total growth inhibition of the OGFr overexpressing clones that was 2.5- to 4.6-fold greater than that for the WT and EV groups, which had 20-24% reductions in cell number with exogenous OGF administration (Figure 7.2C).

**NTX increases cell number in cells overexpressing OGFr**

To understand the effects of opioid receptor blockade with a potent and long-acting opioid receptor antagonist on cells with an abundance of OGFr, 10^-6 M NTX was added to the cultures (Figure 7.2C). Clonal cell lines, as well as WT and EV cells, subjected to NTX exhibited an increase in cell number compared to basal levels. However, the magnitude of increase in cell number was 2.0- to 3.6-fold greater in clonal cell lines with molecular amplification of OGFr and exposed to NTX than in WT or EV cultures receiving NTX (Figure 7.2C).

**OGFr overexpression decreases DNA synthesis without altering cell survival**

To evaluate the mechanism by which an excess of OGFr decreases ovarian cancer cell number, DNA synthesis and cell survival were evaluated. In comparison to the BrdU labeling index of WT (26%) and EV (25%) cultures, the labeling index in clonal cell lines overexpressing OGFr was decreased 59-68% (Figure 7.2D). Examination of
apoptosis (TUNEL) or necrosis (trypan blue) revealed less than 0.1% positive apoptotic or necrotic cells, and these data were comparable for all groups regardless of OGFr expression (data not shown).

**OGF is present but expression levels are unchanged in clonal cell lines**

To examine OGFR levels in cultures overexpressing OGFr, semiquantitative immunohistochemistry for OGFR was performed. OGFR was visible in the cytoplasm with a speckling of immunoreactivity often noted in cell nuclei (Figure 7.3A). Cells processed with secondary antibody only showed no staining (Figure 7.3A inset). OGFR levels as detected by semiquantitative immunofluorescence (mean gray value) were comparable between OGFR-overexpressing cell lines and WT and EV cells (Figure 7.3B).

**Overexpression of OGFR in subcutaneous xenografts reduces tumor incidence, retards tumor appearance, and decreases tumor volume**

Measurable tumors (i.e., > 62 mm$^3$) began to form 4 days following tumor cell inoculation in mice receiving WT or EV cells. One day later, when 90% and 100% of mice inoculated with WT and EV cells, respectively, had measurable tumors, 0% of mice inoculated with OGFR-3 or OGFR-22 cells had measurable tumors (Figure 7.4A). By days 10 and 20, when 100% of mice in the WT and EV groups had measurable tumors, only 10% and 30%, respectively, of mice receiving OGFR-3 cells, and 50% and 80%, respectively, of mice administered OGFR-22 cells, had measurable tumors (Figure 7.4A). By the end of the study (day 32), 40% of mice inoculated with OGFR-3 cells and 90% of mice receiving OGFR-22 cells displayed measurable tumors. An evaluation of the latency to development of measurable tumors revealed latencies of 4.33 ± 0.17 and 4.30 ± 0.15 days for mice administered WT or EV cells, respectively (Figure 7.4B). Animals inoculated with clones OGFR-3 or OGFR-22 that developed measurable tumors had latencies that were 12 and 4 days longer, respectively, than mice inoculated with WT or EV cells (Figure 7.4B). No differences in incidence or latency to form a measurable tumor were detected between WT and EV groups.

Of mice that developed measurable tumors, tumor volumes were decreased 28-87% in mice injected with OGFR-3 cells, and reduced 19-78% in mice inoculated with OGFR-22 cells, beginning on days 11 and 6, respectively; these measurements persisted throughout the duration of the study compared to WT and EV controls (Figure
Comparable tumor volumes were noted in mice receiving WT or EV cells with the exception of day 11 (Figure 7.4C).

Overexpression of OGFr in subcutaneous or intraperitoneal xenografts decreases terminal tumor measurements, without affecting terminal spleen or animal weights

Compared to WT and EV controls on the day of termination (day 32), mice inoculated subcutaneously with clones overexpressing OGFr and developing measurable tumors displayed a visible reduction in tumor size (Figure 7.5A). Terminal tumor volume and tumor weights were decreased 78-99% in mice injected with OGFr-3 or OGFr-22 cells compared to WT and EV controls (Figures 7.5B, C).

In the intraperitoneal xenograft model, mice inoculated with clones overexpressing OGFr (OGFr-3 or OGFr-22) displayed a 95% and 65% reduction, respectively, in the total number of tumor nodules compared to EV controls at the end of the 40 day study (Figures 7.5D,E), reflecting changes in the number of nodules identified on the liver, intestines and stomach. With respect to tumor nodules detected on the spleen, comparable numbers were noted in mice inoculated with OGFr-22 and EV cells; however, there were no nodules on the spleen noted in mice inoculated with OGFr-3 cells (Figure 7.5E).

Total tumor weights in mice receiving intraperitoneal injections of OGFr-3 and OGFr-22 cells were reduced 99% and 69%, respectively, relative to EV controls (Figure 7.5F). Animal and spleen weights were comparable in mice injected either subcutaneously or intraperitoneally with OGFr overexpressing clones relative to mice inoculated with WT or EV cells (data not shown).

OGFr overexpression inhibits DNA synthesis in xenografts without affecting cell survival

Examination of late stage apoptosis in tumors by TUNEL assay revealed similar levels of cell death in mice inoculated with WT, EV, OGFr-3, or OGFr-22 cells (Figures 7.6A, B). With respect to DNA synthesis, comparable rates were noted in tumors from mice receiving WT (31.9 ± 1.2) or EV (30.6 ± 1.5) cells. However, DNA synthesis was reduced in tumors from mice in the OGFr-3 and OGFr-22 groups by 78% and 67%, respectively, compared to WT controls (Figures 7.6C, D).
OGF and OGFr expression in xenografts from mice administered cells with an excess of OGFr

To investigate the distribution and expression of OGFr and OGF in tumors, semiquantitative immunohistochemistry was performed. The location of OGFr was similar in tumors from all groups of mice, with immunoreactivity for this receptor detected in the cytoplasm and a speckling of immunoreactivity noted in cell nuclei (Figure 7.7A). Photodensitometric measurements revealed that OGFr expression was increased 112-146% in mice inoculated with OGFr-3 or OGFr-22 cells, compared to WT and EV controls (Figure 7.7B).

The location of OGF in ovarian tumors was similar in all groups of mice, with immunoreactivity detected in the cytoplasm and a speckling often noted in cell nuclei (Figure 7.7C). Photodensitometric measurements revealed that OGF expression was unchanged in tumors from mice inoculated with cells having an abundance of OGFr compared to WT and EV controls (Figure 7.7D).

**Tumor angiogenesis is reduced in xenografts overexpressing OGFr**

Relative to tumor vessel density in mice inoculated with WT or EV cells, vessel density was reduced 86% and 65% in mice injected with OGFr-3 or OGFr-22 cells, respectively (Figures 7.7E, F).
Discussion

This study is the first to report the stable molecular overexpression of OGFr in a human ovarian cancer cell line, and reveals that upregulation of OGFr markedly inhibits the proliferation of cells in vitro and tumorigensis in vivo. In tissue culture, ovarian cancer cells engineered to have an overexpression of OGFr had decreases in cell number and DNA synthesis, increases in doubling time, and exhibited considerably more modulatory capability in the face of challenges with an opioid agonist (OGF) or antagonist (NTX) compared to WT or EV controls. Xenografts using the subcutaneous route with cells having an abundance of OGFr had increases from control levels in the interval to form a measurable tumor, decreases in the number of animals displaying a measurable tumor, and reductions in tumor volume and weight. With the intraperitoneal route, both the number of metastases and tumor weight were markedly reduced from WT and EV controls. In both in vitro and in vivo investigations, the effects of an excess of OGFr were maintained, indicating that there was no tolerance to the repercussions from amplification of OGFr in these cells.

Evidence of stable overexpression of OGFr in ovarian cancer cells comes from several avenues of experimentation. An increase from control levels (WT, EV) in OGFr protein was detected with semiquantitative immunohistochemistry and Western blotting. The functional capability of the overexpressed OGFr in cellular homogenates to bind to OGF was documented in receptor binding studies, wherein a significant increase from the WT and EV ovarian cancer cells in binding capacity was detected. The overexpression of OGFr in these neoplastic cells did not alter binding affinity of OGF to the receptor, indicating that the processes accompanying the translation of the excess OGFr in these cells was comparable to that in WT cells. Moreover, the binding affinity, as well as binding capacity, in EV cells also was similar to that in WT cells, denoting that the vector did not contribute to any changes in the overexpressed OGFr. Finally, the selection and characterization of multiple clonal cell lines with overexpressed OGFr insured that the outcome of the transfection was a consistent rather than isolated observation. That the additional OGFr had a physiological action in cells that was of greater magnitude than in WT cells, suggests that the downstream pathways (e.g., nucleocytoplasmic transport) remained intact and accommodated the excess OGFr.
To investigate the mechanism by which OGFr overexpression inhibits cell number and tumor progression, cell survival and DNA synthesis were assessed. No changes in the number of apoptotic and/or necrotic ovarian cancer cells with molecular amplification of OGFr were discerned either in tissue culture or in xenografts when compared to WT or EV cells/tissues. Therefore, the reduction in tumor size could not be accounted partially or completely by alteration of cell survival pathways. However, DNA synthesis, both under in vitro and in vivo environments, was markedly depressed in the ovarian cancer cells with an abundance of OGFr compared to WT or EV controls. These results are entirely consistent with previous reports showing that the OGF-OGFr axis serves to maintain the pace of cell proliferation through an inhibitory cascade [7-11], with peptide-receptor interaction targeted to upregulating the cyclin dependent inhibitory kinase pathways [11-14].

The OGF-OGFr axis is known to regulate cell proliferative events with respect to the vascular system, including angiogenesis and repair of vascular injury [39-41]. In the case of overexpression of OGFr in ovarian cancer cells, xenografts were found to have a reduction from control levels in the number of blood vessels associated with these tumors. These data would suggest that the reduction in tumor burden was correlated with a decrease in the vascular supply needed for nutrition. Thus, ovarian tumorigenesis responded to both a direct effect from an excess of OGFr (i.e., a decrease in cell proliferation), and an indirect effect on depressing angiogenesis as a consequence of smaller tumor burden.

As OGF has previously been identified as the opioid that binds to OGFr to inhibit cell proliferation in ovarian cancer [11], the question can be raised as to whether the growth inhibition seen with an abundance of OGFr could be due in part to a compensatory increase in OGF expression. A number of observations in the present study indicate that this is not the case. First, one would predict that if OGF levels increased in proportion to receptor number, then the clonal cell lines with the greatest receptor number would have the highest levels of OGF and hence, the greatest decreases in cell proliferation and tumorigenesis. The data, however, revealed that receptor number alone could not be used as a predictor of cell proliferation in vitro or tumorigenesis in mice. Placing clonal variation aside, all of the cell lines with increased
OGFr were markedly inhibited in cell proliferation and tumorigenesis in contrast to controls. Finally, semiquantitative immunohistochemistry performed on cells and xenografts revealed that OGF expression was unaltered in clonal cell lines with an excess of OGFr compared to controls. Thus, it does not appear that changes in OGF expression are responsible for the observed growth alterations seen with overexpression of OGFr. Moreover, an increase in OGFr number does not signal a downregulation in the required levels of OGF.

To evaluate the total magnitude by which the OGF-OGFr axis can modulate the growth of ovarian cancer cells, clonal cell lines overexpressing OGFr were treated with exogenous OGF or subjected to continuous opioid receptor blockade with NTX. Cultures exposed to OGF or NTX responded with a decrease or increase, respectively, in cell number compared to cohorts treated with vehicle. Cells overexpressing OGFr, however, displayed a greater than 2-fold enhanced response to opioid receptor antagonism, but a comparable response to exogenous OGF compared to WT or EV controls. If one totals the overall magnitude of response to OGF and NTX, growth regulation by the OGF-OGFr axis was 2.5- to 4.6-fold greater in OGFr overexpressing clones than in WT or EV cultures. Thus, the OGF-OGFr axis has a considerable range of modulatory capability in human ovarian cancer cells.

The present observations on the effects of an abundance of OGFr in ovarian cancer cells with regard to cell proliferation and tumorigenesis complement previous reports on the repercussions of molecular manipulation of OGFr. Earlier studies have shown that transient transfection of OGFr cDNA into rat corneal epithelial cells using a gene gun depressed DNA synthesis [42] and wound healing [43]. Additionally, similar to reports in squamous cell carcinoma of the head and neck [24, 44] and pancreatic cancer [23, 45], the present study demonstrates that stable transfection of OGFr cDNA markedly suppressed neoplastic events under both in vitro and in vivo conditions. These data reflect the fundamental nature and biological significance of the OGF-OGFr axis in human ovarian cancer.

Clinically, OGF has been detected by radioimmunoassay in surgical samples taken from human neoplasms of the ovary [26]. The demonstration in the present study that tumor progression is inhibited in mice transplanted with human ovarian cancer cells
overexpressing OGFr indicates that the OGF-OGFr axis is functional in ovarian cancer in vivo. The basal equilibrium of cell replication, which is regulated by endogenous OGF and mediated by OGFr, is disturbed by an abundance of OGFr, with the net result being an overall reduction in cell proliferation. Given that ovarian cancer is the most lethal gynecologic malignancy [4], and that the survival rate for this neoplasia has not improved substantially in decades [3], new strategies to treat this deadly cancer are needed. The findings of this study can be used clinically in designing treatments that capitalize on the body's own processes to restore homeostasis in cell proliferation of ovarian cancer. For example, one could upregulate either the peptide (e.g. by exogenous OGF) and/or receptor (e.g. by gene delivery, imiquimod) to enhance anticancer activity. OGF has been successfully documented to be safe for administration in humans [46] and efficacious in a phase II trial with OGF in pancreatic cancer patients [47].

Conclusion

The present investigation provides insight into the molecular mechanisms of the OGF-OGFr axis as an integral component controlling ovarian cancer cell proliferation and tumorigenesis, and provides evidence that this system can be exaggerated in ovarian cancer to depress the progression of disease. The findings in this study have clinical importance in designing treatment modalities that take advantage of this biological inhibitory axis.
Figures and legends

Figure 7.1. The expression and distribution of OGFr in SKOV-3 cells transfected with an OGFr expression vector. (A) Photomicrographs of untransfected wild-type cells (WT), cells transfected with an empty vector (EV), or clonal cell lines overexpressing OGFr, stained with anti-OGFr antibody (1:200), and taken at the same exposure time. Rhodamine conjugated IgG (1:1000, red) served as the secondary antibody, and nuclei are visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar = 10 µm. (B) Semiquantitative measurement of OGFr staining intensity (mean gray value) from at least 100 cells/group and 3 coverglasses/group. (C) Western blot and densitometric analysis of OGFr normalized to β-actin from 2 independent experiments. (D) Saturation isotherms calculating binding capacity (B_max) of radiolabeled OGF from at least 3 independent assays performed in duplicate. Data for all experiments represent means ± SE. Significantly different from WT at *p<0.05, **p<0.01, or ***p<0.001.
Figure 7.2. Clones overexpressing OGFr are decreased in cell proliferation and can be modulated by OGF and NTX. (A) Growth curves of clonal cell lines, as well as WT and EV cells, from at least 2 aliquots/well and 2 wells/timepoint/cell line. (B) Doubling times of cells calculated from at least 2 growth curves/cell line and analyzed by linear regression. (C) The growth effects of exogenous OGF (10^{-6} M), NTX (10^{-6} M), or an equivalent volume of sterile water (basal level) on WT, EV, and clonal cell lines. Compounds and media were replaced daily. (D) Quantification of DNA synthesis (% BrdU incorporation) in WT, EV, and clonal cell lines for at least 10 fields/coverglass and at least 2 coverglasses/cell line. Values for A, B, and D represent means ± SE; significantly different from WT at *p<0.05, **p<0.01, and ***p<0.001. Values in C are expressed as % growth inhibition or stimulation compared to WT basal levels.
Figure 7.3. The expression and distribution of OGF in SKOV-3 cells transfected with an OGFr expression vector. (A) Photomicrographs taken at the same exposure time of untransfected wild-type cells (WT), cells transfected with an empty vector (EV), or clonal cell lines stained with anti-OGF antibody (1:200). Rhodamine conjugated IgG (1:1000, red) served as the secondary antibody, and nuclei are visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar = 10 μm. (B) Semiquantitative measurement of OGF staining intensity (mean gray value) from at least 100 cells/group and at least 3 coverglasses/group. Data represent means ±
Figure 7.4. OGFr overexpression suppresses tumor progression in mice with subcutaneous xenografts of human ovarian cancer. (A) Incidence of measurable (≥62.5 mm$^3$) tumors at 4, 5, 10, 20, and 32 days following inoculation of mice with 4 x 10$^6$ WT, EV, OGFr-3, or OGFr-22 cells. (B) Latency (in days) to the development of measurable tumors. (C) Measurement of tumor volume (mm$^3$) over time in mice developing measurable tumors. Values represent means ± SE for animals developing measurable tumors. Significantly different from WT at *p<0.05, **p<0.01 and ***p<0.001, and from EV at +p<0.05, ++p<0.01 and +++p<0.001. NS = not significant; dash (−) = insufficient data to perform statistical analyses.
Figure 7.5. Terminal measurements recorded in mice with subcutaneous or intraperitoneal xenografts of human ovarian cancer. (A) Representative images of tumors removed from mice 32 days following subcutaneous inoculation of $4 \times 10^6$ WT, EV, OGFr-3, or OGFr-22 cells. Bar $= 1$ cm. (B) Terminal tumor volume (mm$^3$) in mice developing measurable subcutaneous tumors. (C) Terminal tumor weight (g) in mice with measurable subcutaneous xenografts. (D) Representative images of the peritoneal cavity of mice 40 days following inoculation with $5 \times 10^6$ EV, OGFr-3 or OGFr-22 cells. Bar $= 1$ cm. Arrows indicate tumors. (E) Number of tumor nodules in mice with intraperitoneal xenografts. (F) Terminal total tumor weight (g) in mice with intraperitoneal xenografts. Values represent means $\pm$ SE for all mice developing measurable subcutaneous tumors, and all 8 mice/group in intraperitoneal studies. Significantly different from the WT and/or EV groups by ***$p<0.001$. 
Figure 7.6. Mechanism of tumor growth inhibition by overexpression of OGFr: Effects on apoptosis and DNA synthesis. Tumors were assessed 32 days after mice were inoculated subcutaneously with a) WT, b) EV, c) OGFr-3, or d) OGFr-22 cells. (A) Photomicrographs of TUNEL staining. Negative and positive controls included according to the manufacturer's instruction. (B) Number of apoptotic cells per 0.003 mm$^2$. (C) Photomicrographs of BrdU staining. (D) Quantification of the BrdU labeling. Values for B and D determined from at least 10 fields, 2 sections/mouse, and 2 mice/group. Data represent means ± SE. Significantly different from WT at ***p<0.001. Bar = 20 μm. Arrows indicate positive staining.
**Figure 7.7.** Expression of OGF and OGFr, as well as blood vessel density, in tumors. Tumors were assessed 32 days after mice were inoculated subcutaneously with WT, EV, OGFr-3, or OGFr-22 cells. (A, C) Photomicrographs taken at the same exposure time of tumors stained with antibodies (1:200) to OGFr (A) or OGF (C). Rhodamine conjugated IgG (1:1000) served as the secondary antibody and nuclei are visualized with DAPI. Preparations incubated with secondary antibodies only (insets). (B, D) Semiquantitative measurement of OGFr (B) and OGF (D) staining intensity (mean gray value) from 10 fields from at least 2 sections/tumor with 3 mice/group. (E) Photomicrographs of hematoxylin and eosin staining identifying endothelial lined vessels containing red blood cells. Arrow indicates positive staining. (F), Number of blood vessels per 0.16 mm² determined from at least 10 fields, 2 sections/mouse, and 2 mice/group. Data for all experiments represent means ± SE. Significantly different from WT at ***p<0.001. Scale bar = 10 μm in A and C and 60 μm in E.
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Chapter 8: Stable Underexpression of OGFr

*In Vitro and In Vivo*

Rationale
The importance of OGFr in the regulation of ovarian cancer cell proliferation and tumor growth has been demonstrated thus far through upregulation by NTX treatment, as well as through molecular overexpression studies both *in vitro* and *in vivo*. Upregulation of OGFr markedly inhibits both ovarian cancer cell proliferation and tumor growth and supports treatment modalities for ovarian cancer that amplify OGFr. To date, we have evidence through siRNA knockdown of OGFr in ovarian cancer cells *in vitro* that OGFr is critical to the regulation of cell proliferation. However, we have never evaluated the repercussions of stably underexpressing OGFr on tumor growth *in vivo*. Because the OGF-OGFr axis is a tonically active inhibitory system, it is expected that tumor growth will be accelerated, which is not of clinical value. However, this study is expected to provide proof of principle evidence as to OGFr’s importance, as well as to evaluate whether OGFr expression levels may determine the success or failure in how a patient will respond to OGF treatment in a clinical setting.
Underexpression of the opioid growth factor receptor (OGFr) in human ovarian cancer cells upregulates cell proliferation \textit{in vitro} and accelerates tumorigenesis in mice

Submitted for publication

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Abstract

Background: The opioid growth factor (OGF) and its receptor, OGFr, serve as a tonically active inhibitory axis regulating the proliferation of human ovarian cancer cells. In the present study, we have asked the question as to the repercussions on the progression of this deadly neoplasia when cells are engineered to molecularly underexpress OGFr.

Methods: shRNA constructs were utilized to knockdown OGFr in SKOV-3 cells, and two clonal cell lines were examined. Untransfected wild-type (WT) cells and empty vector (EV) transfected clones served as controls. In tissue culture, the effect of OGFr underexpression on cell number, DNA synthesis, and cell survival was assessed under standard growth conditions, as well as in response to challenge with the opioid agonist OGF or the opioid antagonist naltrexone. Utilizing xenografts, the repercussion of diminishing OGFr in ovarian cancer cells on the course of tumorigenesis was assessed in female nude mice transplanted subcutaneously with WT, EV, or clones engineered to underexpress OGFr, and treated on a daily basis with OGF (10 mg/kg) or an equivalent volume of vehicle (saline).

Results: OGFr protein expression was decreased up to 73% in clones compared to WT and EV controls. OGFr binding assays of clones revealed 50-55% decreases in binding capacity compared to control cells; binding affinity was comparable in all groups. Cell number in clones was increased 33-132%, and doubling times decreased 29-35%, compared to WT and EV cultures. Addition of exogenous OGF or naltrexone did not affect cell number in cultures with silenced OGFr. DNA synthesis of clonal cell lines was increased 136-146% from the WT and EV groups; no changes were noted in cell survival. Nude mice injected subcutaneously with cells underexpressing OGFr had an increased tumor incidence, decreased latency to tumor formation, increased tumor volume, and decreased OGFr expression in tumors compared to WT and EV controls. OGF treatment in mice with WT or EV tumors, but not OGFr underexpressing tumors, inhibited tumor volume, weight, and DNA synthesis.

Conclusions: Collectively, these data demonstrate the critical nature of the OGF-OGFr axis as a determinant of the onset and progression of human ovarian cancer, and
suggest that attenuation of this native regulatory system has an important bearing on the survival of these patients.
Background

With an estimated 225,000 new cases of ovarian cancer worldwide reported each year, resulting in an estimated 140,200 deaths annually [1], this neoplasia is the leading cause of death from gynecological malignancies [2]. Approximately 90% of primary ovarian cancers are epithelial in origin [3], and the majority (75%) of cases are diagnosed in advanced stages (FIGO stage III/IV) [4]. Despite 75-80% of women initially responding to standard treatments (cytoreductive surgery and adjuvant chemotherapy), more than 65% relapse within 12 to 18 months of therapy and, thereafter, all subsequent treatments are palliative [5]. A more profound understanding of the complex biology of this disease represents the most promising avenue to improve the prognosis of ovarian cancer patients [4].

An integral component of the ovarian cancer phenotype is dysregulation of cell proliferation [6]. One native biological regulator of cell replication in normal cells and a wide variety of cancers, including ovarian cancer, is the opioid growth factor (OGF) and its receptor, OGFr [7-12]. Chemically termed [Met^5]-enkephalin, OGF is a constitutively active native opioid peptide that is autocrine produced and secreted, and interacts with OGFr to delay the G1/S interface of the cell cycle by upregulating cyclin-dependent kinase inhibitory (CKI) pathways [12-15] without affecting cell survival [12, 16]. Although OGFr has pharmacological characteristics of classical opioid receptors (recognizes opioids, naloxone reversibility, stereospecificity), this receptor shares no homology with classical opioid receptors at the nucleotide or amino acid level, and has a different cellular localization [7, 17-20]. Regulation of cell proliferation by the OGF-OGFr axis involves nucleocytoplasmic trafficking from the outer nuclear envelope to the nucleus that requires nuclear localization signals and transport by karyopherin β and Ran [18, 21].

OGF has been detected by radioimmunoassay in surgical samples taken from human neoplasms of the ovary [22], and OGFr RNA and protein expression, as well as binding activity, have been documented in human ovarian cancer cells [12]. Using an in vitro model, Donahue and collaborators [12] have identified OGF as the only opioid peptide inhibiting human ovarian cancer cell proliferation, and OGFr was established as the opioid receptor interacting with OGF to maintain the pace of cell replication.
Attenuation of OGF-OGFr interfacing by continuous exposure to the opioid antagonist naltrexone (NTX), neutralization of OGF by antibodies to the peptide, or transient knockdown of OGFr using siRNA, resulted in an increase in ovarian cancer cell number relative to control levels [12, 22]. Furthermore, the addition of OGF in ovarian cancer cultures having a transient knockdown of OGFr did not depress cell proliferation [12, 22].

In the present report we have asked the question as to the repercussions of having a stable underexpression of OGFr in human ovarian cancer cells. We found that persistent molecular underexpression of OGFr increased cell proliferation in vitro and the development of ovarian tumors when cells were subcutaneously inoculated into nude mice. Moreover, in contrast to the inhibition in progression of neoplasia seen with OGF treatment in mice having normal OGFr levels in their tumors, mice with xenografts underexpressing OGFr did not respond to exogenous OGF. These results demonstrate the important role that the OGF-OGFr axis plays regarding the onset and progression of human ovarian cancer.
Methods

Cell culture

The human ovarian cancer cell line SKOV-3 [23] was obtained from the American Type Culture Collection (Manassas, VA), and grown in a humidified atmosphere of 5% CO$_2$/95% air at 37$^\circ$C in RPMI medium supplemented with 1.2% sodium bicarbonate, 10% fetal calf serum, 5,000 U/ml penicillin, 5 µg/ml streptomycin, and 10 mg/ml neomycin.

Transfection and clonal selection

Synthetic double stranded oligonucleotides derived from OGFr siRNA (5’-GATCCGGTCGAGGTGTTTAAAAGCTTCAAGAGAGCTTTTAAACCTCGACCTGA-3’) (Penn State University College of Medicine Core Facility) were cloned into a pSilencer 4.1 – CMV hygro (Ambion, Austin TX) expression vector (OGFr shRNA). SKOV-3 cells were transfected for 24 h in antibiotic free media with OGFr shRNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA) to generate clones. Four h after initiation of transfection, cells were supplemented with serum containing medium. The negative control pSilencer 4.1 – CMV hygro vector (Ambion), with limited homology to human coding cDNAs, was used to generate empty vector (EV) clones. Transfected cells were selected by growth in media containing Hygromycin-B (50,000 mU/ml) (EMB Chemicals, Gibbstown, NJ); 2 clones (SH-6, SH-16) were expanded and maintained in media containing Hygromycin-B. Untransfected wild-type (WT) and EV cells served as controls.

Cell growth

Cells were plated and counted 24 h later (time 0) to determine seeding efficiency. For treatment studies, 10$^{-6}$ M OGF or NTX (Sigma Aldrich, Saint Louis, MO) was added at time 0; media and compounds were replaced daily. Compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. Cells were harvested at designated times, stained with trypan blue, and counted with a hemacytometer. At least 2 aliquots/well from 2 wells/treatment/timepoint were sampled.
Animals

Four week-old athymic nu/nu female mice, purchased from Charles River Laboratory (Wilmington, MA), were housed in pathogen-free isolator ventilated cages in a controlled-temperature room (22-25°C) with a 12-12 h light/dark cycle (lights on 0700-1900). Sterile rodent diet (Teklad, Indianapolis, IN) and water were available ad libitum. All procedures were approved by the IACUC committee of the Pennsylvania State University College of Medicine, and conformed to the guidelines established by the NIH. Mice were allowed 48 h to acclimatize before experimentation.

Tumor cell implantation and treatment

For preliminary tumor burden studies, 3 concentrations (1 x 10^6, 2 x 10^6, or 4 x 10^6) of clonal cell lines stably underexpressing OGFr, (SH-6 and SH-16), as well as EV and WT cells, were injected subcutaneously into the right scapula region (~0.1 ml/mouse) of 3 unanaesthetized mice/group. In the subsequent treatment study, using 8 to 12 mice/group, animals receiving a single concentration (1 x 10^6) of WT, EV, or SH-6 cells were randomly assigned to receive daily intraperitoneal injections of OGF (10 mg/kg) or an equivalent volume of saline. Treatments were initiated when tumors became visible. OGF was dissolved in saline and prepared weekly.

Tumor growth

Mice were weighed weekly and observed daily for the development of visible tumors. The latency to emergence of visible, as well as measurable (i.e. >62.5 mm³), tumors were recorded. For the treatment study, tumors were measured in two dimensions with vernier calipers 3 times/week. Volume was calculated using the formula l x w^2 x π/6 where length (l) is the longest dimension and width (w) is perpendicular to the length [24]. In mice displaying visible, but not yet measurable, tumors, dimensions of tumors were estimated at 3 mm in length and width.

Termination day measurements

Mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and cervical dislocation at 21 days following tumor cell inoculation. To examine DNA synthesis in tumors, mice were injected intraperitoneally with BrdU (100 mg/kg) (Sigma Aldrich) at 6 and 3 h prior to euthanasia [25, 26]. Upon sacrifice, mice were weighed, tumors and spleens removed and weighed, and the lymph nodes, liver, and spleen were examined for the presence of tumors.
examined for metastases. Tumors were processed for immunohistochemistry, Western blotting, TUNEL, and BrdU analysis.

**Semiquantitative immunohistochemistry**

Immunohistochemistry was utilized to evaluate the presence and relative levels of OGF and OGFr in cells and tumor tissue following published procedures [12]. Cells were grown on coverglasses for 72 h, while tumors were excised, frozen in chilled isopentane, and sectioned at 10 μm; preparations were fixed, permeabilized, and stained with antibodies to OGF and OGFr that were generated in our laboratory [27]. To evaluate relative expression levels, images were taken at the same exposure time. In cells, the mean intensity of staining was determined for at least 100 cells/group and 3 coverglasses/group. With respect to tumors, a random sample of 10 fields/section from 2 sections/tumor, and 3 tumors/group, were assessed. Controls were incubated with secondary antibodies only.

**Protein isolation and Western blotting**

Expression of OGFr was evaluated in cells and tumors by Western blotting following published procedures [12]. Cells were sheared with a 25 G needle, while tumor tissue was homogenized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN). Equal amounts of protein (60 μg) were subjected to 15% SDS-PAGE, transferred to nitrocellulose, and probed with an antibody to OGFr (1:200). Optical densities (QuickOne, Sigma Aldrich) were normalized to β-actin (1:5000, Sigma Aldrich). The percent change in expression was calculated by dividing the normalized values to that of WT samples. Means and SE were determined from 2 independent experiments.

**OGFr binding assays**

Log phase cells in culture were assayed for OGFr using custom synthesized [³H]-[Met⁵]-enkephalin (PerkinElmer; Waltham, MA; 52.7 Ci/mmol). Binding assays followed procedures by Donahue et al [12]. Saturation binding isotherms were generated using GraphPad Prism software (GraphPad Prism, La Jolla, CA), and independent assays were performed at least 3 times.
Mechanism of modulated growth: DNA synthesis, apoptosis and necrosis

The effect of underexpressing OGFr on DNA synthesis (BrdU incorporation), apoptosis (TUNEL), and necrosis (trypan blue) was evaluated in cells and tumor tissue. Cells (5 x 10⁴ cells/coverglass) were grown in culture for 72 h; 3 h prior to fixing cells with formalin, 30 μM BrdU was added to these wells [12]. Tumors from mice receiving BrdU on the day of sacrifice were fixed in formalin overnight, processed in paraffin, and sectioned at 10 μm. Preparations were either stained with an antibody to BrdU (1:200, Invitrogen) [12, 25, 26], or processed for TUNEL according to the manufacturer’s instruction (Trevigen, Gaithersburg, MD). Necrosis was ascertained in all growth experiments. In cells, the proportion of those positive for BrdU or TUNEL staining was determined for at least 500 cells/coverglass on 2 coverglasses/group. In tumors, the proportion of BrdU positive cells, and number of cells positive for TUNEL, in a 0.015 mm² area were determined from at least 10 random fields around the periphery of each tumor, utilizing at least 2 sections/tumor and 2 tumors/group.

Statistical analysis

Tumor incidence was analyzed by the Chi square test. All other data were analyzed using one-way analysis of variance (ANOVA), with subsequent comparisons made with the Newman-Keuls tests (GraphPad Prism). In some cases, data were evaluated by unpaired t-tests. P-values <0.05 were considered significant.
Results

Establishment and characterization of the OGFr shRNA clones

Two SKOV-3 clones, SH-6 and SH-16, stably transfected with OGFr shRNA were expanded and characterized by semiquantitative immunohistochemistry, Western blotting, OGF receptor binding, and growth (Figures 8.1, 8.2). In all studies, comparisons were made to (and between) WT and EV cells.

OGFr protein is underexpressed in transfected cell lines

Semiquantitative immunohistochemistry of OGFr revealed a marked decrease in OGFr immunofluorescence (mean gray value) in clonal cell lines SH-6 (23%) and SH-16 (27%) relative to WT cells (Figures 8.1A, B). For all cultures, OGFr was visible in the cytoplasm, and a speckling of immunoreactivity was often noted in cell nuclei. Cells processed with only secondary antibody showed no staining (Figure 8.1A, inset). Western blot analysis of SH-6 and SH-16 clonal cell lines indicated that the 62-kDa band of OGFr, standardized for loading with actin, was decreased 73% and 56%, respectively, relative to WT cells (Figure 8.1C). No differences in OGFr expression were observed between WT and EV groups.

Underexpression of OGFr downregulates receptor binding capacity

To further characterize the OGFr underexpressing clones, OGFr binding capacity (B_max) and binding affinity (K_d) were determined. Binding assays of radiolabeled [Met^5]-enkephalin revealed specific and saturable binding in the nuclear fraction of all cells. Binding capacity for clonal cells was markedly reduced compared to those of WT (3.8 ± 0.2) and EV (3.4 ± 0.2) groups, with binding capacities of 1.9 ± 0.2 and 1.7 ± 0.2 recorded in SH-6 and SH-16 clones, respectively (Figure 8.1D). Binding affinity did not differ between groups (data not shown).

Underexpression of OGFr upregulates cancer cell proliferation

The repercussion of underexpressing OGFr was determined by evaluating cell growth over 96 h. Cell number was increased in SH-6 (48-132%) and SH-16 (33-88%) cultures, compared to EV and WT controls (Figure 8.2A). Calculation of doubling times based on multiple growth curves revealed decreases in clonal cell lines SH-6 (35%) and SH-16 (29%) relative to WT and EV controls (Figure 8.2B). No differences in doubling times were noted between cells in WT and EV cultures (~ 39 h).
Exogenous OGF or NTX have no effect on cell proliferation in cultures underexpressing OGFr

The ability of exogenous OGF (10^{-6} M) or NTX (10^{-6} M) to modulate cell number in clonal cell lines underexpressing OGFr was evaluated. Following 96 h of treatment, cell number was reduced in WT (18%) and EV (22%) cultures administered OGF, and increased in WT (25%) and EV (26%) cells treated with NTX, compared to WT and EV cells subjected to sterile water (Figure 8.2C). In contrast, clonal cell lines SH-6 and SH-16 had a comparable number of cells regardless of treatment with OGF, NTX, or sterile water (Figure 8.2C).

OGFr underexpression increases DNA synthesis without altering cell survival

To evaluate the mechanism by which OGFr underexpression increased cell number, DNA synthesis and cell survival were evaluated. Compared to the labeling index of WT (19%) and EV (22%) cultures, clones SH-6 and SH-16 had 136% and 146%, respectively, more cells incorporating BrdU (Figure 8.2D). Examination of apoptosis (TUNEL) or necrosis (trypan blue staining) in SH-6 and SH-16 clonal cell lines revealed less than 0.1% positive cells for apoptosis or necrosis; these data were comparable to that of WT and EV groups (data not shown).

OGF is present but expression levels unchanged in clonal cell lines underexpressing OGFr

OGF was visible in the cytoplasm, with a speckling of immunoreactivity observed in cell nuclei (Figure 8.3A). No staining was detected in cells processed with secondary antibody only (Figure 8.3A, inset). OGF immunofluorescence (mean gray value) did not differ between groups (Figure 8.3B).

Preliminary tumor burden study: Underexpression of OGFr in human ovarian cancer xenografts accelerates tumor appearance

An initial evaluation of latency to the development of measurable tumors (i.e., ≥ 62.5 mm^3) revealed that mice receiving 1 x 10^6 or 2 x 10^6 EV cells had latencies of 26.7 ± 3.6 and 23.5 ± 2.5 days, respectively (Figures 8.4A, B). Mice inoculated with 1 x 10^6 SH-6 or SH-16 cells had latencies that were 21 and 14 days shorter (i.e. days 5 and 12), respectively, than mice receiving 1 x 10^6 EV cells. Animals administered 2 x 10^6 SH-6 or SH-16 cells had latencies that were 22 and 7 days shorter than mice inoculated...
with $2 \times 10^6$ EV cells (Figures 8.4 A, B). Comparable latencies were noted in mice injected with $1 \times 10^6$ or $2 \times 10^6$ WT or EV cells (Figures 8.4A, B). Latencies to tumor development were similar in animals inoculated with $4 \times 10^6$ WT, EV, SH-6, or SH-16 (data not shown).

**Mice with xenografts underexpressing OGFr have increased tumor incidence, accelerated tumor appearance, increased tumor volumes, and fail to respond to OGF**

In order to i) confirm the findings in the preliminary tumor burden study, ii) determine the effects of molecular underexpression of OGFr on tumor incidence and tumor volume, and iii) assess whether OGF could modulate tumors underexpressing OGFr, mice inoculated with $1 \times 10^6$ WT, EV or SH-6 cells were injected on a daily basis with OGF (10 mg/kg) or an equivalent volume of saline when tumors became visible. In contrast to WT and EV groups receiving saline, which contained no mice with measurable tumors on day 4, 58% of mice in the SH-6 group and receiving saline had measurable tumors (Figure 8.5A). On day 5, when 83% of the mice in the SH-6 group had measurable tumors, only 12% and 8% of mice in the WT and EV groups, respectively, had tumors. By day 7, when 100% of mice injected with SH-6 cells and saline had measurable tumors, only 25% of WT and 16% of EV mice had measurable tumors. On days 19 and 21, 100% of the mice in the WT and EV groups, respectively, had measurable tumors, in contrast to the SH-6 group that reached a 100% incidence on day 7. The latency to appearance of a measurable tumor for mice injected with WT (11.9 ± 1.5 days) or EV (13.0 ± 1.5 days) cells and exposed to saline was comparable, but was significantly decreased by 9 days in mice injected with SH-6 cells and treated with saline (Figure 8.5B). Evaluation of tumor volumes in mice injected with SH-6 cells and subjected to saline revealed that tumor volumes were elevated 41-267% on days 4-14 relative to mice inoculated with WT cells and receiving saline (Figure 8.5C).

Compared to saline administered WT or EV controls, mice inoculated with WT or EV cells and treated with OGF had reduced tumor volumes (38-60%) beginning on day 14 (Figure 8.5C), and decreased terminal tumor weights (32-55%) (Figure 8.5D). Tumor volumes and terminal tumor weights were comparable in mice inoculated with SH-6 cells that were treated with OGF relative to mice injected with SH-6 cells receiving
saline (Figures 8.5C, D). Terminal spleen and body weights were comparable regardless of the cell line inoculated, as well as with respect to OGF or saline treatment (data not shown).

**OGF inhibits DNA synthesis without affecting cell survival in xenografts from mice with normal OGFr levels**

On the day of sacrifice, comparable BrdU labeling indexes were noted in tumors taken from WT (24.6 ± 1.9), EV (26.8 ± 1.9), and SH-6 (27.0 ± 2.0) groups subjected to saline (Figure 8.5E). However, in mice inoculated with WT or EV cells and treated with OGF, DNA synthesis in tumors was reduced up to 38% from saline administered controls (Figure 8.5E). In contrast, comparable levels of DNA synthesis were recorded in mice receiving SH-6 cells and administered OGF or an equivalent volume of saline. Examination of apoptosis in xenografts revealed comparable levels in mice inoculated with WT, EV, or SH-6, regardless of exposure to OGF or saline (data not shown).

**OGFr underexpression is maintained in xenografts**

To examine OGFr distribution and expression in the transition from *in vitro* to *in vivo* environments, semiquantitative immunohistochemistry and Western blot were performed on tumors taken from mice inoculated with WT, EV, or SH-6 cells. The location of OGFr was similar in all groups, with immunoreactivity detected in the cytoplasm and a speckling noted in cell nuclei (Figure 8.6A). Tumors processed only with secondary antibody showed no staining (Figure 8.6A inset). Compared to the WT group, OGFr expression in SH-6 xenografts was decreased as noted by semiquantitative immunohistochemistry (35%) (Figure 8.6B) and Western blot (67%) (Figures 8.6C, D).

**OGF expression is unchanged in xenografts underexpressing OGFr**

To ascertain OGF distribution and expression in xenografts with decreased OGFr, semiquantitative immunohistochemistry was performed using an antibody to OGF. OGF was visible in the cytoplasm, with some immunoreactivity often noted in cell nuclei (Figure 8.6E). Tumors processed with only secondary antibody showed no staining (Figure 8.6E inset). OGF distribution and immunofluorescence (mean gray value) did not differ between groups (Figures 8.6E, F).
Discussion

This study is the first to report the stable underexpression of OGFr in a cancer cell line, and reveals that diminishing OGFr in human ovarian cancer cells markedly affects cell proliferation and tumorigenesis. In vitro, attenuation of OGFr resulted in increases in cell number and DNA synthesis, along with decreases in doubling times. Cultures with a deficit of OGFr did not respond to challenges with an opioid agonist (OGF) or antagonist (NTX), in contrast to WT or EV cultures that were inhibited or increased by exposure to OGF and NTX, respectively. These findings in tissue culture were extended into animals using subcutaneous tumor transplantation, and revealed that OGFr underexpression increases tumor incidence, decreases the latency to development of measurable tumors, and increases tumor volumes. Thus, under both in vitro and in vivo conditions, the effects of a loss of OGFr were maintained, indicating that there was no tolerance or compensation to the repercussions of decreasing OGFr in these cells.

Knockdown of OGFr was confirmed in cells and tumor tissue by a combination of semiquantitative immunohistochemistry and Western blot analysis. Binding capacity of OGFr was markedly reduced in clonal cell lines underexpressing OGFr in comparison to WT and EV cells. However, no changes in binding affinity were noted, demonstrating that there was no compensatory action (i.e., increase in binding affinity) of the decreased OGFr. Furthermore, the vector did not contribute to OGFr knockdown, as WT and EV cells were similar in OGFr expression and binding, as well as in growth (in vitro and in vivo). Additionally, the characterization of two clones insured that the outcomes of transfection were not related to an aberrancy of only a singular clone, but rather were representative of the repercussions from underexpression of OGFr.

Although the methodology utilized in these studies diminished OGFr by approximately 70%, this extent of underexpression did have a significant effect on function. For example, cells with a reduction in OGFr were increased in BrdU labeling index by more than 1.4-fold from WT cells. Moreover, tumor incidence was notably increased in mice inoculated with cells underexpressing OGFr, with 100% of these mice having measurable tumors within 1 week compared to almost 3 weeks for the WT and EV groups. Therefore, the magnitude of underexpression achieved in this study was
sufficient to evoke a physiological effect \textit{in vitro} and \textit{in vivo}. Whether a complete knockout of OGFr in ovarian cancer cells would even further hasten cell proliferative events and tumorigenesis in comparison to controls remains to be investigated.

Because of the marked acceleration in proliferation of cells underexpressing OGFr, our strategy for tumor studies was to use a low tumor burden in order to provide a window to assess changes for comparison to the WT and EV groups. Because of our methodology of using a lower tumor burden, a plateau effect of tumor size was observed. This was evident in the time to reach maximal tumor volume, with mice inoculated with cells underexpressing OGFr reaching this measure on day 4, in contrast to WT and EV groups that took 5 times longer to attain a comparable tumor size.

Because OGF has been identified as the opioid peptide that binds to OGFr to repress ovarian cancer cell proliferation [12], one can ask whether the growth acceleration recorded with a reduction in OGFr could be the result, at least in part, to a compensatory decrease in OGF. The data gathered in this study do not support this hypothesis. First, OGF levels as measured by semiquantitative immunohistochemistry were similar in cultures and tumors underexpressing OGFr to those of WT and EV cells/tissues. Second, even when exogenous OGF was added to cells in culture with diminished OGFr, as well as animals with xenografts of these cells, cell number and tumor volumes remained elevated compared to cells/animals in the WT and EV groups. Moreover, mice with underexpression of OGFr and receiving OGF were comparable in tumor progression to mice with a diminishment of OGFr and treated with vehicle. This was distinct from the marked decreases in cell number and tumor volumes in WT and EV groups exposed to OGF wherein reductions of up to 60% were recorded. These data demonstrate that there is a loop between OGF and OGFr that is critical to regulating cell number in ovarian cancer.

To examine the mechanism by which OGFr underexpression accelerates cell number in tissue culture and tumorigenesis, cell survival and DNA synthesis were monitored. No changes in the number of apoptotic or necrotic ovarian cancer cells with a molecular attenuation of OGFr were discerned \textit{in vitro} or \textit{in vivo} in contrast to WT and EV cells/tissues. Thus, alteration in cell survival pathways did not account for the acceleration in cell number and tumor progression. However, DNA synthesis under \textit{in
vitro conditions was markedly elevated in cells with a deficiency in OGFr compared to WT or EV controls. In vivo, DNA synthesis was comparable at the time of termination in all experimental groups treated with vehicle, when tumors were of similar size. This was most likely due to the employing of a low tumor burden which had the net effect of all tumors reaching a maximum and comparable size. However, DNA synthesis was notably inhibited in tumor tissue harvested from mice in the WT and EV groups that were treated with OGF relative to the same groups receiving saline. These results showing an OGF repression of tumorigenesis in ovarian cancer are consistent with previous reports in other cancers documenting a mechanism targeted to cell proliferation [28-31].

The present findings reveal an acceleration of cell proliferation and tumorigenesis with knockdown of OGFr, demonstrating the tonic activity of the OGF-OGFr axis in human ovarian cancer. A continuously active loop between native OGF and OGFr has been recorded earlier in tissue culture of ovarian cancer with i) continuous blockade of OGF-OGFr with NTX, ii) neutralization of native OGF with an antibody to OGF, and iii) transient knockdown of OGFr with siRNA, all markedly increasing cell proliferation [12, 32]. Additional evidence gathered in these experiments found that diminishment of OGFr in ovarian cancer disengaged and attenuated peptide-receptor interfacing under in vitro conditions, thereby rendering cells insensitive to the growth modulatory effects of exogenous OGF (i.e., inhibition) and NTX (i.e., stimulation). Moreover, tumors generated from cells underexpressing OGFr were not responsive to the effects of OGF administration. Thus, molecular engineering of an underexpression of OGFr has extremely specific and focused repercussions on the replication of ovarian cancer cells, as well as on tumorigenic events.

Conclusions

The present study clearly reveals that the OGF-OGFr axis is a fundamental biological pathway that impacts the course and progression of human ovarian cancer. This raises the question as to the repercussions of potential alterations (e.g., mutation, processing) of OGF and/or OGFr on the growth of ovarian tumors. Moreover, since the OGF-OGFr system involves other biological processes (e.g., nucleocytoplasmic transport), dysfunction of one or more of these pathways could impair regulation of cell
proliferation - and tumorigenesis - in patients with ovarian cancer. These findings that the OGF-OGFr axis is of vital importance to advancement of ovarian cancer, implies that strategies to harness this peptide-receptor system offers a distinct means to restore homeostatic conditions. Modulation of the OGF-OGFr axis can be achieved by a variety of means, including i) administration of exogenous OGF [28-31], ii) the use of imiquimod to upregulate OGFr [33], and iii) treatment with low dose naltrexone (LDN) which upregulates both OGF and OGFr and, after drug is no longer present, allows for a robust functional effect by reducing cell proliferation [34-37]. Of course, as documented herein, these strategies are dependent on a functioning OGF-OGFr system.
Figures and legends

**Figure 8.1.** The expression and distribution of OGFr in SKOV-3 cells transfected with OGFr shRNA. (A) Photomicrographs of untransfected wild-type cells (WT), cells transfected with an empty vector (EV), or clonal cell lines SH-6 and SH-16 stained with antibodies (1:200) to OGFr; photomicrographs were taken at the same exposure time. Rhodamine conjugated IgG (1:1000) served as the secondary antibody (red) and nuclei were visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar = 10 μm. (B) Semiquantitative measurement of OGFr staining intensity (mean gray value) from at least 100 cells/group utilizing 3 coverglasses/group. (C) Western blot of OGFr and densitometric analysis normalized to β-actin from 2 independent experiments. (D) Saturation isotherms calculating binding capacity (B_max) of radiolabeled OGF for at least 3 independent assays performed in duplicate. Data in B, C, and D represent means ± SE. Significantly different from WT at **p<0.01 and ***p<0.001.
Figure 8.2. Clones underexpressing OGFr are increased in cell proliferation and cannot be modulated by OGF or NTX. (A) Growth curves of SH-6, SH-16, WT, and EV cells from at least 2 aliquots/well and 2 wells/time point. (B) Doubling times calculated from at least 2 growth curves/cell line and analyzed by linear regression. (C) The growth effects of exogenous OGF (10^{-6} M), NTX (10^{-6} M), or an equivalent volume of sterile water (basal level) on WT, EV, SH-6, and SH-16 cell lines. Compounds were replaced daily. (D) Quantification of DNA synthesis (% BrdU incorporation) in cells for at least 10 fields/coverglass from 2 coverglasses/cell line. Media was changed daily in all experiments. Values for A, B, and D represent means ± SE; significantly different from WT at *p<0.05 and ***p<0.001. Values in C are expressed as % growth inhibition or stimulation, compared to WT basal levels.
**Figure 8.3.** The expression and distribution of OGF in SKOV-3 cells transfected with OGFr shRNA. (A) Photomicrographs of untransfected wild-type cells (WT), cells transfected with an empty vector (EV), or SH-6 and SH-16 cell lines stained with an antibody to OGF (1:200). All photomicrographs were taken at the same exposure time. Rhodamine conjugated IgG (1:1000, red) served as the secondary antibody, and cell nuclei were visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar = 10 µm. (B) Semiquantitative measurement of OGF staining intensity (mean gray value) from at least 100 cells/group and 3 coverglasses/group. Data represent means ± SE.
Figure 8.4. Preliminary tumor burden study: OGFr underexpression decreases the latency to the development of measurable tumors. (A, B) Latency (days) to the development of a measurable (≥62.5 mm³) tumor in mice receiving subcutaneous inoculation of 1 x 10⁶ (A) or 2 x 10⁶ (B) WT, EV, SH-6, or SH-16 cells. Data represent means ± SE for 3 animals/group. Significantly different from mice receiving WT cells at **p<0.01, and ***p<0.001.
**Figure 8.5.** Repercussion of underexpressing OGFr on tumor progression and response to OGF treatment. Mice were inoculated subcutaneously with $1 \times 10^6$ WT, EV, or SH-6 cells and treated daily with OGF (10 mg/kg) or an equivalent volume of saline when tumors became visible. (A) Incidence of measurable ($\geq 62.5$ mm$^3$) tumors at 4, 5, 7, 19, and 21 days following tumor cell inoculation in saline treated mice. (B) Latency (days) to the development of measurable tumors in mice administered saline. (C) Tumor volume in mice forming visible tumors that were treated with OGF or an equivalent volume of saline. (D) Terminal tumor weight. (E) Quantification of DNA synthesis (% BrdU incorporation) in tumors on the day of sacrifice. Data in A, B, C, and D represent means $\pm$ SE for 8-12 animals/group. Values in E represent means $\pm$ SE determined from at least 10 fields at the periphery of the tumor, utilizing at least 2 sections/tumor and 2 tumors/group. Significantly different from WT at *p<0.05, **p<0.01 and ***p<0.001, from EV at +p<0.05, ++p<0.01 and +++p<0.001, and from the WT group treated with OGF at ^p<0.05, ^^p<0.01 and ^^^p<0.001.
Figure 8.6. Distribution and expression of OGFr and OGF in subcutaneous tumors. Tumors were assessed 21 days after mice were inoculated with WT, EV, or SH-6 cells. (A, E) Photomicrographs of tumors stained with antibodies (1:200) to OGFr (A) or OGF (E). All photomicrographs were taken at the same exposure time. Rhodamine conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei visualized with DAPI. Preparations incubated with secondary antibodies only (A, E insets). Bar = 10 μm. (B, F) Semiquantitative measurement of OGFr (B) and OGF (F) staining intensity (mean gray value) from 10 fields/section, 2 sections/tumor, and 3 mice/group. (C) Western blot of OGFr expression in mice inoculated with SH-6, WT, and EV cells. (D) Densitometric analysis of Western blots normalized to β-actin from 2 independent experiments. Data represent means ± SE. Significantly different from WT at **p<0.01 and ***p<0.001.
References


Chapter 9: Overall Discussion
9. **Overall Discussion**

9.1. **Summary**

The central hypothesis of this thesis is that ovarian cancer progression is dependent on the repercussions of the interaction between OGF and OGFr, and that modulation of this axis may be utilized for therapeutic purposes. Utilizing a combination of *in vitro* and *in vivo* techniques, I have identified the role and mechanism of the OGF-OGFr axis in human ovarian cancer, as well as determined its relevance as a potential therapeutic. The studies performed here demonstrate that the OGF-OGFr pathway regulates ovarian cancer cell proliferation and support treatment modalities for ovarian cancer that upregulate this axis. A number of novel findings are presented in this thesis:

**In human ovarian cancer cells *in vitro***:

- OGF and OGFr are present
- Endogenous OGF is constitutively produced, secreted, and tonically active
- Exogenous OGF inhibits cell number in a dose-dependent, serum-independent, reversible, and receptor-mediated manner
- Establishment of a tissue culture model of LDN using short term NTX administration demonstrated that LDN inhibits cell number independent of immune function and systemic factors by upregulating OGF and OGFr at the translational level
- The opioid peptide mediating LDN’s action is OGF
- The opioid receptor mediating LDN and OGF’s action is OGFr, not μ, δ, or κ opioid receptors
- Without affecting cell survival, OGF and LDN inhibit cell proliferation by targeting the p16 and/or p21 CKI pathways
- Growth effects of OGF, but not NTX, require protein and RNA synthesis
- Stable genetic overexpression of OGFr inhibits cell proliferation without affecting cell survival to decrease cell number and increase doubling times
• Stable genetic underexpression of OGFr accelerates cell proliferation without affecting cell survival to increase cell number and decrease doubling times
• OGF or LDN can be combined with standard of care chemotherapies taxol or cisplatin for an additive inhibitory effect on growth

In human ovarian cancer in vivo:
• OGF or LDN treatment alone inhibited tumor growth in mice with established subcutaneous xenografts by inhibiting cell proliferation and tumor angiogenesis without inducing apoptosis
• OGF treatment in combination with taxol or cisplatin, and LDN treatment in combination with cisplatin but not taxol provided an additive inhibitory effect on tumor growth in mice with established subcutaneous xenografts
• OGF and LDN inhibit tumor growth in the more clinically relevant intraperitoneal xenograft model of human ovarian cancer by inhibiting cell proliferation and tumor angiogenesis without inducing apoptosis
• Stable genetic overexpression of OGFr in tumor cells decreased tumor incidence, increased latency to tumor formation, and decreased tumor volumes
• Stable genetic underexpression of OGFr in tumor cells increased tumor incidence, decreased latency to tumor formation, and increased tumor volumes
• OGF treatment failed to inhibit tumor growth in mice with normal OGFr levels but xenografts stably underexpressing OGFr

In summary, the data from these studies have supported that the OGF-OGFr axis is present in human ovarian cancer and serves as a regulatory mechanism to inhibit cell proliferation in a non-toxic manner. This axis functions both in vitro and in vivo, and treatments that upregulate this axis (OGF, LDN) can be combined with standard of care chemotherapies for enhanced therapeutic benefit. The pre-clinical studies presented in this thesis support therapeutic modalities that amplify OGF-OGFr activity for the treatment of human ovarian cancer, and warrant testing of OGF and LDN in the clinic.
9.2. Comparison of Agents Modulating the OGF-OGFr Axis

9.2.1. Lack of Tolerance

A number of important observations stemming from these studies should be noted. First, no tolerance, at least in the time frame of the current studies, was noted in response to treatment modalities that upregulated the OGF-OGFr axis. That is, tumors did not escape from the regulation of the OGF-OGFr axis when this axis was elevated by administration of OGF or LDN, or genetic manipulation to overexpress OGFr. Mice with established xenografts treated with OGF or LDN for 37 and 35 days, respectively, had tumor volumes that were significantly inhibited throughout the duration of the study. Similarly, mice inoculated with cells overexpressing OGFr had tumors that remained markedly depressed in volume over a 32 day period. It may be argued that the downregulation of OGFr seen in tumors from mice treated with OGF is a mechanism by which tolerance could be induced; however, the persistent inhibitory effect on tumor volume throughout the duration of the study indicates that enough OGFr remained to be able to mediate the inhibitory effects of the exogenous OGF supplied.

The lack of tolerance observed with regulation of tumor growth by the OGF-OGFr axis is very different to what is typically seen in humans treated with standard of care chemotherapies. Acquired resistance to chemotherapy is a major obstacle to successful ovarian cancer treatment (1). Many human cancers are naturally resistant to anticancer drugs (2); however, with ovarian cancer, tumors initially respond well to drugs at first but then eventually treatment fails because the cancer becomes resistant (3). Mechanisms of acquired tolerance can include interruption of drug uptake or activation, increased drug efflux or catabolism, mutations in target genes, or increased repair of DNA damage (2, 4). Mechanisms by which cells develop resistance to cisplatin include inhibition of drug uptake, an increase in the production of cellular thiols which block the formation of DNA adducts, enhanced replicative bypass of cisplatin-DNA adducts, alterations in the concentration of regulatory proteins, and an increase in the repair of cisplatin-DNA adducts (5). For taxol, alterations in cellular transport of taxol through overexpression of the membrane P-glycogen which functions as a drug efflux pump, or alterations in transcriptional and posttranscriptional modifications of microtubules that alter the binding affinity of microtubules to taxol, are thought to be
important in acquired resistance of cancer cells to this agent (6). The lack of tolerance seen with upregulation of the OGF-OGFr axis may be due to the fact this system serves as a native biological regulator of cell proliferation and is not toxic.

9.2.2. Lack of Toxicity and Amelioration of Toxicity

Another important observation noted in these studies, is that mice treated with agents that upregulate the OGF-OGFr activity (OGF, LDN, genetic overexpression of OGFr) were not toxic, with body and spleen weights unaltered compared to appropriate controls. This is in contrast to standard of care treatments for ovarian cancer which are associated with debilitating side effects, including neurotoxicity, nephrotoxicity, alopecia, and fatigue (7). Not only were OGF and LDN not toxic, but they actually reduced the toxicity associated with cisplatin use when administered in combination with this agent. Importantly, the anticancer effects of cisplatin were not attenuated by co-administration with LDN or OGF; in fact, an enhanced anti-tumor activity was seen in animals treated with cisplatin in combination with OGF or LDN. The protection afforded by OGF or LDN from the side effects of cisplatin may allow higher doses of cisplatin to be administered to improve the therapeutic efficacy of this agent. This would be highly advantageous, as chemotherapy success is often limited by an intrinsic resistance of cancer cells as well as unacceptable toxicity at higher dosages. In the case of cisplatin, the risk of severe nephrotoxicity hinders the use of higher doses that would maximize its antineoplastic effects (8, 9). The possibility of increasing the concentration of drugs without an accompanying increase in cytotoxicity would be important therapeutically (7).

These studies are not the first to note the protective effects of the OGF-OGFr axis. [Met⁵]-enkephalin has been shown to mediate cardiac protection in an opioid receptor mediated manner against ischemic damage both in vitro (10, 11) and in vivo (12). Furthermore, a synthetic opioid met-enkephalin analog has been reported to protect against gastric damage induced by oral administration of necrotizing agents in rats (13). This protective effect could be blocked by NTX, indicating it was mediated by an opioid receptor (13). [Met⁵]-enkephalin has also been shown to have a protective effect against cysteamine-induced duodenal ulcers in rats (14). Finally, in mice with xenografts of SCCHN that were treated with a toxic regimen of taxol, OGF treatment was shown to increase mouse survival (15).
9.2.3. Effects of OGF-OGFr Modulation on the Expression of OGF and OGFr

The present study has demonstrated three strategies by which OGF-OGFr activity can be increased to inhibit ovarian cell proliferation and tumor growth: treatment with OGF, administration of LDN to produce intermittent opioid receptor blockade, and stable molecular overexpression of OGFr. Even though these three approaches all similarly inhibit cell proliferation and tumor growth in a non-toxic manner by upregulating the overall signal of OGF-OGFr, the effects on the expression of OGF and OGFr in tumor tissue are varied.

First, in contrast to the increase in OGFr expression observed with LDN treatment as well as with genetic upregulation of OGFr, OGF administration actually reduced the expression and binding of OGFr in a self limiting fashion. Importantly, even with the reduction in OGFr expression seen with prolonged OGF treatment, tumors retained their sensitivity to exogenous OGF. This is in contrast to the insensitivity to exogenous OGF seen in cells genetically manipulated to underexpress OGFr. The reason for this discrepancy may be due to the extent to which OGFr was decreased. OGF treatment reduced OGFr binding to 3.649 fmol/mg protein, while in the case of molecular underexpression, OGFr binding was reduced to 1.74 fmol/mg protein. Additionally, the timing and degree of permanence for which OGFr was reduced in these scenarios could factor into whether cells/tumors respond to exogenous OGF. In the case of OGF treatment, OGFr levels were downregulated in response to excess OGF, while in the case of OGFr molecular underexpression, cultures were passaged a number of times and conditioned to underexpress OGFr well in advance of OGF administration. Finally, with OGF treatment, the reduction in receptor levels was not permanent, with mice that stopped receiving OGF treatment (for 2 weeks following 37 days of OGF) having OGFr levels that returned to levels observed in tumors prior to OGF treatment (Figure 9.1). This lack of permanence is in contrast to the constant underexpression of OGFr seen with molecular underexpression of OGFr.

Additionally, differences were noted as to the expression levels of OGF in tumor tissue taken from mice treated with LDN to induce intermittent opioid receptor antagonism, compared to mice having a molecularly induced overexpression of OGFr.
Pharmacologic manipulation of OGF-OGFr with LDN upregulated both OGFr and OGF, in contrast to genetic upregulation of OGFr, which had no effect on the expression levels of OGF in cells or tumors, as assessed by semiquantitative immunohistochemistry. In the case of LDN treatment, OGFr levels were initially normal before they were upregulated in response to opioid receptor blockade, while in the case of OGFr molecular overexpression, cultures were passaged a number of times and conditioned to stably overexpress OGFr. Importantly, it was demonstrated that the magnitude to which cell number could be inhibited was increased when cultures stably overexpressing OGFr were treated with exogenous OGF, indicating that the levels of endogenous OGF were not sufficient to occupy all of the upregulated OGFr. Similarly, cells treated with short term NTX, (the equivalent of LDN) were able to be further inhibited by concurrent treatment with OGF, suggesting that even though OGF was upregulated by NTX treatment, the levels were not sufficient to saturate OGFr to mediate maximal growth inhibition.

Differences were additionally noted as to the expression levels of OGF in two of the studies where mice were administered OGF. Despite 37 days of OGF treatment in the established subcutaneous xenograft model, cellular levels of OGF were unchanged on the day of sacrifice, as assessed by semiquantitative immunohistochemistry. In contrast, tissue OGF levels were found to be elevated, as assessed by semiquantitative immunohistochemistry, following 35 days of OGF treatment in the intraperitoneal xenograft model. This disparity is most likely due to the timing of when mice were euthanized with respect to their last OGF treatment. In the subcutaneous model where OGF levels were unchanged following OGF treatment, mice were euthanized approximately 24 h after their last OGF treatment; in the intraperitoneal model where OGF levels were increased with OGF treatment, mice were euthanized within 2-4 h of the last OGF injection. These findings suggest that OGF levels may only remain elevated for a transient period of time following OGF administration.

It is interesting to note that in several instances, regulation of OGFr has been shown at the translational but not transcriptional level. For example, in patient samples of SCCHN, OGFr protein but not mRNA levels were found to be markedly reduced in tumor tissue compared to normal epithelium, with tumor margins having intermediate
levels of OGFr (16). An additional study evaluated the regulation of OGFr expression in the progression of SCCHN by evaluating small, medium and large tumors in mice transplanted with SCCHN, and found a reduction in OGFr protein with increasing tumor size; however, no changes in OGFr mRNA were detected (17). The finding in the present report, that LDN upregulates OGFr protein but not mRNA levels, is in agreement with these studies, indicating regulation of this receptor at the translational level. With this being said, OGFr mRNA has been shown to be differentially expressed during rat development (18), as well as in various organs taken from mice (19). Additionally, treatment of SCCHN or pancreatic cancer cells with imiquimod was reported to upregulate OGFr at both the mRNA and protein level (20). Thus, OGFr is able to be regulated at both the transcriptional and translational level; however, LDN treatment only upregulates OGFr protein without affecting OGFr mRNA levels.

9.2.4. Efficacy

All three mechanisms to upregulate OGF-OGFr activity (treatment with OGF or LDN, or molecular overexpression of OGFr) significantly inhibited ovarian cancer tumor growth and did so by inhibiting DNA synthesis and reducing tumor angiogenesis without inducing apoptosis. All three treatment modalities were evaluated in subcutaneous xenografts as well as in the more clinically relevant intraperitoneal xenograft model of human ovarian cancer. In the subcutaneous xenograft models, tumor volumes were inhibited up to 50% by OGF treatment, 48% by LDN, and 80% to 95% by OGFr genetic overexpression. It is, however, unfair to directly compare the effects of OGFr overexpression with OGF or LDN treatment in the subcutaneous xenograft model, because OGFr was stably overexpressed prior to tumor cell implantation, whereas OGF and LDN were tested on established tumors. In the intraperitoneal xenograft model, however, OGF or LDN treatments were initiated at the time of tumor cell implantation, which is more similar to when OGFr was overexpressed. In this model, OGF and LDN treatments reduced the number of tumor nodules by 41% and 38%, respectively, while OGFr overexpression inhibited the number of tumor nodules by 65% to 96%, depending on the clonal line evaluated. As for terminal tumor weights, OGF and LDN treatments reduced tumor weights by 68% and 46%, respectively, while OGFr overexpression inhibited tumor weights by 68% to 99%, once again depending on the clonal line.
examined. Treatment with OGF prevented intraperitoneal tumor formation in 1 of 8 mice, while stable overexpression of OGFr (in one of the two clonal lines evaluated) completely inhibited tumor formation in 3 of 8 mice examined 40 days later. In contrast, 8 of 8 mice treated daily with LDN developed tumors. Although all methods to upregulate OGF-OGFr activity were effective at inhibiting ovarian tumor growth, these results indicate that they can be ranked in terms of effectiveness, with stable overexpression of OGFr being the most effective, followed by OGF biotherapy, followed by LDN treatment.

Combination studies evaluating the efficacy of OGF or LDN biotherapy and standard of care chemotherapies (taxol, cisplatin) also support that OGF was more effective than LDN when added to these agents. OGF was able to be combined for an additive inhibitory effect with cisplatin or taxol, while LDN was only effective with cisplatin, but not taxol, for an improved benefit. However, based on the finding that LDN treatment upregulates OGFr, in contrast to OGF administration which reduced OGFr expression, LDN may in fact may prove to be more beneficial than OGF in patients having an underexpression of OGFr protein (e.g. patients with SCCHN). Additionally, the in vitro studies of this thesis suggest that OGF may be able to be combined with LDN for an additive inhibitory effect on ovarian cancer.

9.3. Limitation of Xenografts in Nude Mice

Nude mice, discovered in 1966, are an inbred strain with a missing or damaged thymus gland, resulting in a deficient immune system (21). These mice also happen to lack fur. Because they are athymic, nude mice cannot generate mature T lymphocytes, which are essential to the adaptive immune response. They are unable to mount many types of immune responses, including antibody formation that requires CD4+ T helper cells, cell-mediated immune responses which require CD4+ and/or CD8+ T cells, delayed hypersensitivity responses which require CD4+ T cells, killing of virus-infected or malignant cells which require CD8+ cells, and graft rejection which requires CD4+ and CD8+ T cells (22). The nude mouse was a major breakthrough for cancer research, because it allowed human tumors to be studied in laboratory animals. Before the discovery of the nude mouse, human tumors were grafted and grown in immune-privileged sites, such as the anterior chamber of the eye, the brain, and the cheek.
pouch; however, these locations were inconvenient and tumors were eventually rejected (23). With a very limited immune system, the nude mouse is able to receive many tissue and tumor grafts from many different species without initiating a rejection response. Despite the limitations associated with using immunocompromised mice, they are vital to cancer research as they allow the study of novel therapeutics on human tumors, which retain their original characteristics.

An additional criticism of many xenograft models is the relevance of the location of tumor formation. The advantage of subcutaneous xenografts is that tumor progression is easy to track. An intraperitoneal xenograft model of human ovarian cancer, where human ovarian cancer cells are injected intraperitoneally and disseminated throughout the abdomen of mice is very similar to human disease, with cell accumulation and tumor growth commonly occurring on the liver, stomach, spleen, and walls of the peritoneal cavity. The limitations of the intraperitoneal model are the difficulty in tracking tumor burden because animals need to be euthanized each time, and hence, the expense incurred with such a model. The present study utilized a combination of both models (subcutaneous and intraperitoneal) to obtain the preclinical information necessary to determine the role of the OGF-OGFr axis on ovarian tumor growth.

9.4. Immunity

9.4.1. Role of Immunity in Ovarian Cancer

Various components of the innate and adaptive immune response are able to mediate tumor cell destruction; however certain immune cell populations can also produce a pro-tumor microenvironment that favors tumor growth and the development of metastases. Multiple observations in clinical studies support a role for the immune system in controlling ovarian tumor growth and progression (24–28). In fact, some of the strongest evidence linking anti-tumor immunity and cancer in general has been made in ovarian cancer (24).

The first evidence of the role of immunosurveillance against human ovarian cancer was the identification of CD3⁺ tumor-infiltrating lymphocytes (TIL's) which correlate positively with patient survival (25). In that study, patients whose tumors lacked TIL's had 5 year overall survival rates of 4.5% and 5 year progression free
survival rates of 8.7%, while patients containing TILs had 5 year overall survival rates of 38%, and 5 year progression free survival rates of 31% (25). That same study also demonstrated that the presence or absence of TIL’s correlated with the extent of residual tumor (25). Another study confirmed these findings, demonstrating improved overall survival (>60 months versus 29 months) among patients with epithelial ovarian cancer with higher versus lower counts of CD3+ TILs (26).

Additional reports have revealed a pro-tumor role of T regulatory cells (Tregs CD4+CD25+FOXP3+) on the progression of ovarian cancer. Sato et al demonstrated that patients with higher frequencies of CD8+ TILs had improved survival compared to patients with lower frequencies (55 versus 26 months) (27). Demonstrating that CD4+ TILs negatively influence the beneficial effects of CD8+ TILs, patients having a high versus low ratio of CD8+/CD4+ TILs had median survival rates of 74 months and 25 months, respectively, (27). The unfavorable effects of CD4+ T cells on prognosis were hypothesized to be due to Treg suppressor T cells (27). Curiel et al provided the first direct evidence that Treg suppressor TILs (CD4+CD25+FOXP3+) correlate to a poor clinical outcome (25.1 higher risk of death) in epithelial ovarian cancer, demonstrating that the percentage of Tregs was higher in stage II–IV disease than in stage I disease (28). Importantly, the Tregs identified had regulatory function, suppressing the proliferation of CD3+CD25- T cells as well as IFN-γ and IL-2 production in vitro (28).

These studies all strongly suggest that the T cell immune response against ovarian cancer is a significant and independent prognostic factor, and highlight the possibility that a treatment causing a favorable anti-ovarian cancer immune response could result in improved clinical outcome.

9.4.2. OGF-OGFr axis and Immunity

Based on the literature suggesting that a T cell immune response against ovarian cancer is a significant prognostic factor, a limitation of the present study is that any potential effects of the OGF-OGFr axis on T-cell mediated effects on ovarian tumor growth cannot be evaluated using nude mice. A number of lines of evidence, however, indicate that the immune system is not necessary for OGF-OGFr to mediate its inhibitory effects on tumors. First, the fact that OGF or LDN administration, as well as OGFr overexpression inhibit ovarian cell proliferation in tissue culture indicates that this
axis does not require an upregulation of the immune system or other systemic factors. Furthermore, the studies demonstrating the efficacy of OGF or LDN administration, as well as OGFr overexpression on ovarian cancer cells transplanted into nude mice, indicates that this axis does not require T-cell mediated immunity for its inhibitory actions on tumor growth. In fact, OGF appears able to mediate its inhibitory actions directly on the cancer cells themselves, based on the demonstration that OGF’s inhibitory actions were lost in mice with normal OGFr levels that were transplanted with cancer cells stably underexpressing OGFr. This is in contrast to the inhibitory effect of OGF treatment in mice having normal levels of OGFr in their tumors. Although these studies collectively indicate that OGF-OGFr does not require an immune mediated response for its inhibitory action on ovarian tumor growth, it cannot be concluded that this axis does not have an effect on immune cells present in the tumor microenvironment of either an immunocompromised or immunocompetent creature.

In fact, the effects of endogenous opioids, including OGF, on various cells of the immune system have been evaluated by a number of investigators with conflicting results reported. Natural Killer cells (NK cells) are a type of cytotoxic lymphocyte that constitutes a major component of the innate immune system and is known to be present in nude mice (29). They are well known to play a major role in the rejection of tumors and cells infected by viruses by inducing apoptosis through the release of small cytoplasmic granules of perforin and granzyme. A number of investigators have demonstrated that short term OGF treatment in vitro increases NK cell activity in human peripheral blood mononuclear cells (PBMCs) taken from normal patients (30-32) as well as patients with cancer (33). Similarly, NK cell activity was shown to be increased in PBMC’s taken from C57BL/6 mice treated with daily OGF for 1, 3, 7, or 14 days (34). In nude mice, however, studies from our laboratory have indicated that 28 days of daily OGF treatment had no effects on NK cell activity in the peripheral blood of mice and reduced NK cell activity in splenic lymphocytes (35). Based on the reported lack of increase in NK cell activity seen in nude mice with OGF treatment (35), as well as the loss of OGF inhibitory activity in mice with tumors underexpressing OGFr, that otherwise have normal OGFr levels, it is unlikely that manipulation of the OGF-OGFr
axis, by treatment with OGF or LDN or molecular overexpression of OGFr, inhibited tumor growth through a mechanism targeted to an upregulation of NK cell activity.

The role of endogenous opioids, including OGF, on the proliferative activity of two other immune cell types involved in the adaptive immune response, T and B lymphocytes have similarly been evaluated by a number of investigators, once again with conflicting results. With regard to T cells, a number of investigators report that OGF administration increased T lymphocytes (34, 36-39), while another group of investigators describe an inhibitory effect of OGF on T lymphocytes (40-43). In contrast, other groups report a lack of inhibitory or stimulatory effects of OGF on T lymphocytes (44-51). Studies from our laboratory found that mitogen induced T lymphocytes are suppressed by the OGF-OGFr axis with a mechanism targeted to cell proliferation (52). As for the role of OGF on B cells, several groups reported an increase in B cell proliferation following OGF administration (34, 53), while other groups did not observe any change in B lymphocyte proliferation following exposure to this opioid peptide (54, 55). An additional laboratory actually reported gender related differences in B lymphocyte proliferation following exposure to OGF (56). Studies in our laboratory; however, found that OGF treatment markedly reduced mitogen induced B cell proliferation without affecting cell survival (57).

Based on the clinical observations demonstrating the importance of the immune system in controlling ovarian tumor growth and progression (25-28), and the demonstrated inhibitory effects of the OGF-OGFr axis on NK cell activity (35), as well as T lymphocyte (52) and B lymphocyte (57) proliferation, it could be argued that upregulation of the OGF-OGFr axis would not be a good treatment strategy for ovarian cancer. However, it should be noted that the studies in our laboratory evaluating the effects of OGF on NK cell activity were in nude mice without tumors, and the experiments on T and B cell proliferation were performed in response to PHA and LPS, respectively, mitogen activation in vitro. The role of the OGF-OGFr axis on NK cell activity, as well as T and B cell proliferation, in the ovarian tumor microenvironment are unknown.
9.5. Clinical Correlations

9.5.1. Enkephalinases and Enkephalinase Inhibitors

A number of studies have suggested that two peptidases known to be involved in the degradation of OGF, CD10 and CD13, play a role in the pathogenesis of ovarian cancer (58-69). First described by Greaves et al in 1975, CD10, also known as neutral endopeptidase (NEP), is a cell surface metalloendopeptidase that functions to reduce cellular response to peptide hormones by regulating local peptide concentrations (70, 71). CD10 is capable of efficiently degrading a number of bioactive peptides including [Met⁵]-enkephalin to reduce its biological activity (72). CD10 expression has been shown to be upregulated in serous borderline and well differentiated serous, endometrioid, and clear cell carcinomas compared with poorly differentiated carcinomas, suggesting that CD10 may be involved in tumor differentiation (58). The authors hypothesized that loss of CD10 in high grade carcinomas may contribute to growth factor-mediated progression of tumor cells (58). Another group reported that CD10 was expressed in the stroma of borderline and malignant ovarian tumors, and that stromal CD10 expression was reduced with increasing histological grades, implying that this peptidase plays a role in the biology of neoplastic transformation through degradation of specific peptide substrates (59). This same group later examined the repercussions of overexpressing CD10 in ovarian cancer cells and found a reduction in proliferation and tumor growth, and thereby proposed a potential role for this enzyme as a suppressor of ovarian carcinoma (60).

Perhaps more relevant to the present work of this thesis are the findings on the role of CD13 in ovarian cancer. CD13, also known as Aminopeptidase N (APN), is a transmembrane ectopeptidase that similarly functions to reduce cellular response to peptide hormones by regulating local peptide concentrations including [Met⁵]-enkephalin (33). A number of studies have indicated that CD13 plays an important role in tumor progression by regulating processes such as cell-cell contact, proliferation, tumor invasion, and metastasis, and have led to the hypothesis that inhibition of CD13 could be an effective strategy for treatment of cancer (61-63). Interestingly, the activity of a
soluble form of CD13 was found to be elevated in malignant ascites from ovarian cancer patients (64).

Three separate studies have indicated that inhibition of CD13 inhibits the growth of human ovarian cancer. In one study, Gao et al reported that LYP, a novel bestatin dimethylaminoethyl ester which potently inhibits CD13 expression (65), reduced the proliferation of human ovarian cancer cells \textit{in vitro}, and delayed tumor formation and reduced tumor sizes \textit{in vivo} (66). In a separate study, CIP-13F, a more potent inhibitor of CD13 than bestatin was evaluated for its effects on ovarian cancer (67). CIP-13F is a cyclic-imide peptidomimetic compound designed to fit the active pocket of CD13 (68). CIP-13F was found to inhibit proliferation, invasion, and migration of ES-2 human ovarian cancer cells \textit{in vitro} as well as delay tumor formation and reduce tumor sizes \textit{in vivo} (67). Finally, Terauchi et al reported a positive correlation between CD13 and the migratory potential of ovarian cancer cell lines, demonstrating a significant decrease in the proliferative and migratory potential of ovarian cancer cells following siRNA to reduce CD13 expression or bestatin administration to reduce CD13 activity (69). Furthermore, this study also demonstrated that inhibition of CD13 using bestatin decreased peritoneal dissemination and prolonged survival of mice with intraperitoneal xenografts of ovarian cancer (69).

A number of investigators have reported that CD13 expression is also related to the chemosensitivity of ovarian cancer. Using a panel of ovarian cancer cells in culture, Yamashito et al demonstrated a negative correlation between CD13 expression and chemosensitivity to taxol (73). They subsequently found that inhibition of CD13 using siRNA or bestatin significantly increased the sensitivity of ovarian cancer cells to taxol \textit{in vitro}, and combination of bestatin with taxol in an intraperitoneal xenograft model of ovarian cancer increased survival time compared with taxol treatment alone (73). Additionally, using a subcutaneous xenograft model, this group showed that intratumoral injection of CD13 siRNA sensitized tumors to taxol treatment (73). This report that CD13 activity may contribute to chemoresistance, possibly through proteolytically modifying peptides involved in anti-apoptotic signaling and/or their precursors, are consistent with a study by van Hensbergen et al, which demonstrated that CD13 overexpressing ovarian cancer cells were less sensitive to cisplatin in a
subcutaneous xenograft model of ovarian cancer (74). However, in contrast to the findings that a reduction in CD13 inhibits ovarian tumor growth (66, 67, 69), van Hensberen and colleagues, reported that CD13 overexpression reduced the growth rate of ovarian cancer cells in vitro (74).

These studies indicating that inhibition of CD13 using enkephalinase inhibitors such as bestatin are efficacious for the treatment of ovarian cancer are important to the studies presented in this thesis, because CD13 is known to degrade OGF, reduce its half life, and therefore reduce its signaling potential. It is possible that the effects on tumor growth seen with downregulation of CD13 may to an extent involve an upregulation of OGF activity. In fact, preliminary experiments in SKOV-3 cells utilizing a mixture of the enkephalinase inhibitors bestatin, captopril, and thiorphan, which have the potential to increase the half life of OGF in tissue culture from 2.8 h to 23.3 h (75), markedly reduced cell number (Figure 9.2). Furthermore, the combination of this enkephalinase inhibitor mixture with exogenous OGF reduced cell number to greater extent than OGF or enkephalinase inhibitors alone (Figure 9.2). Modulation of the OGF-OGFr axis by administration of exogenous OGF or LDN, as well as molecular overexpression of OGFr may be able to be combined with enkephalinase inhibitors in vivo for improved preclinical and clinical benefit.

9.5.2. Increased Copy Number at 20q13

The genetic amplification or loss of growth altering genes is believed to play a key role in the development of human malignancy. A number of studies have implicated one or more genes at 20q13, the location where OGFr maps in humans, as having an important role in ovarian tumor growth and progression. Approximately 20% to 30% of ovarian tumors (preferentially advanced staged tumors) have an increase in the copy number of the 20q13 locus, and this variance in copy number is believed to have prognostic relevance (76-78). The functional role of this increase in copy number is unknown; however, it is an interesting potential connection between the OGF-OGFr axis and human ovarian cancer.

9.5.3. p16 and p21 Expression

Based on the finding in this thesis that the OGF-OGFr axis targets and requires the p16 and/or p21 CKI pathways for its inhibitory action on ovarian cancer, another
clinical significance of this study is that p16 or p21 could potentially be utilized as biomarkers in predicting the response of patients with ovarian cancer to agents that upregulate OGF-OGFr activity. This would allow responders and non responders to OGF-OGFr modulating therapies to be predicted in advance of treatment in an effective and economic manner. A number of studies have actually evaluated the prognostic significance of p16 and p21 expression in ovarian cancer (79-84). Surowiak et al found that decreased p16 expression is an unfavorable prognostic factor, characteristic of cases with overall decreased survival as well as resistance to chemotherapy (79). Similarly, Kommoss et al determined that patients with p16 negative ovarian tumors have a significantly worse prognosis than patients with tumors containing p16 (80). In a subsequent study, Goto et al investigated p16 expression in ascites using liquid – based cytology as a novel prognostic marker for advanced ovarian cancer, and found that overall survival was markedly better in immunopositive cases, compared to immunonegative cases (81). As for p21 expression, decreased levels in ovarian cancer patient samples are also typically associated with poor prognosis. Bali et al reported reduced overall survival and shorter progression free survival times for patients with low p21 expression (82), while Plisiecka-Halasa et al demonstrated that overall survival was positively associated with high p21 expression; p21 expression was additionally found in tumors to serve as a positive predictor of platinum sensitivity (83). Low p21 expression was similarly reported by another group to be a marker of poor overall survival (84).

Based on these reports of poor prognoses of patients with low expression of p16 or p21, the clinical implications for OGF-OGFr in inhibiting ovarian cancer proliferation are profound, as this axis functions by way of an upregulation of p16 and/or p21.

Additionally, since our results demonstrated that the OGF-OGFr axis inhibit ovarian cancer progression by upregulating p16 or p21 levels, other compounds which increase p16 or p21 expression, such as Indole-3-carbinol (I3C) which increases p16 levels (85), or TGF-β which increases p21 levels (86), should be considered for the treatment of ovarian cancer. Furthermore, it is possible that agents that upregulate p16 and/or p21 activity could be combined with OGF for a potential synergetic effect on cell growth.
Our study is not the first study to indicate that upregulation of p16 and/or p21 has potential use for the treatment of ovarian cancer. Adenovirus mediated transfection of p16 in cell lines of ovarian cancer that are p16 null (SKOV-3) as well as in cultures that contain normal p16 (OVCA420) suppressed growth anywhere from 75-85% (87). In an additional study, mice with intraperitoneal xenografts of Hey A8, SKOV-3 or 2774 cell lines that were treated with p16 by intraperitoneal injection of an adenovirus vector had increased survival rates relative to control mice which received an empty vector (88). Similarly, upregulation of p21 by transfection of p21 cDNA into either SKOV-3 or OVCAR-3 human ovarian cancer cells led to a reduction in cell growth, as well as enhanced susceptibility to cisplatin treatment (89). Furthermore, administration of a zinc-citrate compound CIZAR to human ovarian cancer cells was shown to inhibit proliferation through an upregulation of p21 (90).

9.6. OGF Has Strong Potential for Use in the Clinic

The results from this thesis demonstrate for the first time the efficacy of OGF treatment, alone and in combination with standard of care chemotherapies, for the treatment of ovarian cancer. The experimental paradigm of the present study involved exposure of mice to OGF when tumors were established (visible) but small. This regimen markedly reduced tumor volumes and terminal tumor weights. Thus, in a clinical setting, OGF treatment could be foreseen as a tool for ovarian cancer patients with established ovarian cancer as an adjuvant therapy following tumor resection to reduce tumor burden of residual tissue as well as metastases.

An interesting observation that may be of relevance to the efficacy of OGF treatment is that expression of the enkephalinase CD13 has been reported to be less pronounced in samples obtained from secondary cytoreductions following chemotherapy, as opposed to samples obtained from primary laparotomies (91). This could be important in determining the timing for when OGF would be most efficacious in the treatment of ovarian cancer, as CD13 degrades OGF. OGF treatment may actually be more effective in patients following chemotherapy and secondary cytoreductive surgery than in patients newly diagnosed with ovarian cancer.

The demonstration in the present study that OGFr expression and binding is reduced following chronic OGF treatment may have important ramifications on whether
an individual receiving OGF should stop OGF treatment. As long as OGF treatment continues, tumor burden appeared to be inhibited despite the downregulation of OGFr. However, it may be that if an individual stops receiving OGF treatment, there may be a rebound effect where tumors actually grow faster than controls until OGFr levels return to normal. It should be noted that OGFr levels did increase to pre-OGF treatment levels when examined 2 weeks after OGF administration was terminated (Figure 9.1). This is important because, even if an individual stops taking OGF, it is not expected that a possible rebound effect will last longer than this period of time.

In a phase I trial, OGF was administered either intravenously or subcutaneously with escalating doses of OGF to determine the maximal tolerated dose, and was documented to be safe for administration in humans (92). During that study, two subjects had resolution of liver metastases and one patient showed regression of the pancreatic tumor itself. Subsequently, a prospective phase II open labeled clinical trial of 24 subjects who had previously failed standard chemotherapy for advanced pancreatic cancer were treated with OGF and evaluated for clinical benefit, tumor response, quality of life, and survival (93). In that study, OGF biotherapy improved the clinical benefit and prolonged survival in patients with pancreatic cancer by stabilizing disease or slowing progression (93). Trials for OGF in patients with SCCHN and hepatocellular carcinoma are underway (personal communication, D. Goldenberg, E. Kimchi). The reported safety and efficacy of OGF in the clinic indicate that OGF could be easily transitioned from preclinical studies (presented in this thesis), to the bedside for the treatment of human epithelial ovarian cancer.

9.7. LDN Has Strong Potential for Use in the Clinic

The results from this thesis demonstrate for the first time the efficacy of LDN treatment, alone and in combination with standard of care chemotherapies, for the treatment of ovarian cancer. The experimental paradigm of the present study involved exposure of mice to LDN when tumors were established (visible) but small. This regimen markedly reduced tumor volumes and terminal tumor weights. Thus, in a clinical setting, opioid modulation by way of opioid antagonists could be foreseen as a tool for ovarian cancer patients with established ovarian cancer as an adjuvant therapy following tumor resection to reduce tumor burden of remaining tissue and metastasis.
Anecdotal evidence has suggested that LDN may be a beneficial therapy for a variety of autoimmune diseases, including psoriasis, Crohn’s disease, and multiple sclerosis, as patients with these diseases report a decrease in disease progression and increases in quality of life when using off-label LDN for their ailments. In a pilot human study, 89% of patients with Crohn’s disease who received LDN (4.5 mg/day) responded to treatment, with 67% of those individuals experiencing complete disease remission as well as improvement in quality of life as measured by a survey (94). A pilot trial has additionally be conducted to examine the effects of LDN in 40 patients with multiple sclerosis, and reported that LDN is safe, well tolerated, and reduced spasticity without affecting fatigue, depression, and quality of life (95). Cree and coworkers have also found that LDN was well tolerated and serious adverse health issues did not occur in patients with multiple sclerosis (96). In an additional study, Sharafeddinadeh et al reported that LDN was safe but a longer duration trial was needed to determine the efficacy of LDN for multiple sclerosis (97).

A limited number of published reports exist as to the use of LDN in patients with cancer. Berksen et al report the reversal of signs and symptoms of a single patient with B-cell lymphoma treated with only LDN (98). Later in 2009, this same group described 4 case studies where cancer patients were treated with a combination of LDN and alpha-lipoic acid. In that study, the first patient was alive and well 78 months after initial presentation, the second was alive and well 39 months after presenting with adenocarcinoma of the pancreas with metastases to the liver, the third demonstrated no evidence of disease by PET scan following 5 months of therapy after initially presenting with adenocarcinoma of the pancreas with liver metastases, and the fourth who had a history of B-cell lymphoma and prostate adenocarcinoma and presented with pancreatic cancer as well as liver and retroperitoneal metastases, had no signs of cancer by PET scan following 4 months of treatment (99). Based on the clinical reports for the use of LDN in autoimmune diseases, and the published case reports of cancer patients treated with LDN, it appears that the transition from bench to bedside is feasible when designing studies using LDN.

There are several advantages of LDN therapy for the treatment of ovarian cancer over the standard of care chemotherapies or OGF biotherapy. The first involves the
route of administration. NTX is orally effective (100), compared to the standard of care chemotherapy or OGF infusions, where patients must travel to a cancer center to receive treatments. Second, the patent for NTX expired in 1985; therefore the cost of NTX would be much lower than the standard of care chemotherapies or OGF biotherapy (101). Additionally, similar to OGF biotherapy, no toxic side effects were noted in the clinical studies evaluating LDN (94-97), as well as in the present study in mice treated with LDN. This is in contrast to the debilitating side effects associated with cisplatin and taxol chemotherapy (7). Finally, because LDN upregulates OGFr expression, in contrast to OGF which reduces OGFr expression, LDN may prove to be a better treatment for patients having an underexpression or defect of OGFr in their tumors.

9.8. Future Directions

The demonstration in the present study that OGFr expression and binding is reduced following chronic OGF treatment may have important ramifications on whether an individual receiving OGF should stop OGF treatment. Despite the downregulation of OGFr, this study demonstrated that tumor progression was inhibited as long as OGF treatment continued. In light of this decrease in OGFr expression, studies should be performed to evaluate the potential of a rebound following cessation of OGF treatment, as well as explore the timing and extent for which OGFr expression is modulated by OGF treatment. It should be noted that OGFr protein levels, as determined by semiquantitative immunohistochemistry and receptor binding techniques, were returned to normal levels following a 2 week period of no OGF treatment (Figure 9.1). This is important because, even if an individual stops taking OGF, it is not expected that the potential rebound effect will not last longer than this period of time. Strategies to prevent a possible rebound could involve treatment with LDN (to upregulate OGFr levels), or a slow tapering off of OGF treatment.

Based on the reported clinical importance of T cells in the ovarian tumor microenvironment, as well as the inhibitory effects of the OGF-OGFr axis on mitogen induced T cell proliferation, it may be important to evaluate the effects of OGF-OGFr axis modulating agents (OGF, LDN) on T cell activity using an immunocompetent mouse with ovarian cancer. Quinn et al have recently described an immunocompetent
syngeneic mouse model of epithelial ovarian cancer that could be utilized for such studies (102). Although the OGF-OGFr axis directly inhibits ovarian cancer cells in the absence of T cells (tissue culture, nude mouse studies), this axis may also have an immunomodulatory function in the tumor microenvironment that contributes to its antitumor action. A preferential inhibition of Treg cells over CD8+ T cells in the tumor microenvironment, for example would have the potential to contribute positively to an antitumor response.

Additional studies should also be undertaken to evaluate whether the OGF-OGFr axis modulating agents OGF and LDN can be combined with each other for enhanced therapeutic benefit in the treatment of ovarian cancer. In vitro studies suggest this to be the case, with an additive inhibitory effect seen in cultures treated with a single application of short term NTX (the equivalent of LDN in tissue culture) with daily OGF treatment. The results in tissue culture suggest that although both OGF and OGFr are upregulated by LDN, the degree of OGF present is not sufficient to produce a maximal inhibitory response. Whether this phenomenon would hold in vivo in either a subcutaneous or intraperitoneal xenograft model of ovarian cancer warrants testing.

Furthermore, studies should be performed to determine whether the OGF-OGFr axis modulating agents OGF and LDN can be combined with enkephalinase inhibitors, which prolong the half life of OGF, for enhanced therapeutic benefit. The literature documenting that inhibition of CD13 is beneficial at inhibiting the progression of ovarian cancer would support the combination of OGF of LDN with inhibitors of CD13. Glavas-Obrovac et al have documented that a mixture of the enkephalinase inhibitors bestatin, captopril and thiorphan increase the half life of OGF in tissue culture from 2.8 h to 23.3 h (75). I have performed preliminary studies with this mixture of enkephalinase inhibitors alone and in combination with OGF treatment on ovarian cancer cells in vitro and found that the enkephalinase inhibitors alone markedly inhibited SKOV-3 cell number. Furthermore, the combination of this enkephalinase inhibitor mixture with OGF inhibits cell number to a greater extent than OGF or enkephalinase inhibitors alone (Figure 9.2). Additional studies should be performed to evaluate the mechanism of this additive inhibitory effect in vitro, as well as pursue these studies in vivo to see if these agents
can be combined for enhanced therapeutic benefit using animal models of ovarian cancer.

Finally, clinical testing of OGF and LDN for efficacy in patients with ovarian cancer is warranted. The results of this thesis have demonstrated that the OGF-OGFr axis is present in human ovarian cancer, and support modalities that upregulate OGF and/or OGFr. Upregulation of OGF-OGFr signaling by treatment with OGF or LDN, as well as molecular overexpression of OGFr may serve as a potential therapeutic for the treatment of ovarian cancer. Importantly, OGF biotherapy, and LDN treatment can be combined with standard of care chemotherapies for a more efficacious treatment. The preclinical data presented in this thesis support studies to test OGF and LDN in the clinic, both alone and in combination with standard of care therapies, for the treatment of human ovarian cancer.
9.9. Figures and Legends

Figure 9.1. Effect of stopping OGF treatment on OGFr expression and binding. Mice with SKOV-3 subcutaneous xenografts were treated when tumors became visible with OGF (10 mg/kg) or an equivalent volume of saline on a daily basis. Following 37 days of treatment, a subset of mice were euthanized, while another set stopped receiving treatments for additional 2 weeks, at which point the experiment was terminated and the remaining mice were euthanized. Tumors collected from mice sacrificed on days 37 (Saline, OGF) and day 51 (Saline Stopped, OGF Stopped) were processed for immunohistochemistry and receptor binding assays to assess OGFr expression and binding. (A) Photomicrographs taken at the same exposure time of tumors stained with antibodies (1:200) to OGFr. Rhodamine conjugated IgG (1:1000) served as the secondary antibody and nuclei are visualized with DAPI. Preparations incubated with secondary antibodies only (insets). Bar = 10 μm. (B) Semiquantitative measurement of OGFr staining intensity (mean gray value) from 10 fields from at least 2 sections/tumor with at least 3 mice/group. (C) Saturation isotherms calculating the binding capacity (Bmax) of OGFr in xenografts for radiolabeled OGF from at least 2 independent assays performed in duplicate. Data represent means ± SE. Significantly different from saline treated controls at *p<0.05 and ***p<0.001.
**Figure 9.2.** Effect of enkephalinase inhibitors on SKOV-3 cell growth alone and in combination with OGF treatment. Growth of SKOV-3 cells treated with a mixture of the enkephalinase inhibitors bestatin (10 μM), captopril (10 μM) and thiorphan (30 μM), OGF (10^{-6} M), a combination of the enkephalinase inhibitors plus OGF, or an equivalent volume of sterile water (Co) over a 144 h period. Administration of this regimen of enkephalinase inhibitors has been reported to increase the half life of OGF in tissue culture from 2.8 h to 23.3. Compounds were added 24 h after seeding 10,000 cells/well, and media and compounds were replaced daily. Data represent means ± SE for at least 2 aliquots/well from at least 2 wells/group. Significantly different from Co at ***p<0.001, from OGF treated cultures at ^^^p<0.001, and from enkephalinase inhibitor administered cultures at +p<0.05 and +++p<0.001.
9.10. References


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