Gynecologic Oncology 122 (2011) 382–388

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

The opioid growth factor (OGF) and low dose naltrexone (LDN) suppress human ovarian cancer progression in mice

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ARTICLE INFO

Article history:
Received 23 February 2011
Accepted 9 April 2011
Available online 30 April 2011

Keywords:
Ovarian cancer
Opioids
Enkepalins
Xenografts
Opioid growth factor
Low dose naltrexone

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 was 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 an 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.

Materials 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, 5000 units/ml penicillin, 5 μg/ml streptomycin, and 10 mg/ml neomycin.

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doi:10.1016/j.ygyno.2011.04.009

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, unanesthetized mice were injected intraperitoneally with SKOV-3 cells (5×10^6/mouse) following a 48 h acclimation period.

Within 1 h of tumor cell inoculation, three groups of mice were randomized and began 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, subsets of mice were 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 was 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 [^{3}H]-[Met^5]-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 were 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 (Fig. 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 and heart), and ascites was not observed in any group. Compared to the total number of nodules detected in mice administered with saline (39.4 ± 2.8), animals treated with OGF or LDN displayed a 42% and 39% reduction, respectively (Fig. 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 (Fig. 1B). With regard to tumor nodules on the spleen, comparable numbers were noted in all groups (Fig. 1B). Relative to the cumulative tumor weight recorded in saline controls, tumor weight was reduced in mice treated with OGF (69%) and LDN (46%) (Fig. 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 (Fig. 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 (Fig. 2B). The distribution of OGFr immunoreactivity in tumor tissue was detected in the cytoplasm and often lightly scattered in cell nuclei (Fig. 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 (Fig. 3B). Tumors processed with only secondary antibody showed no staining (Figs. 2A and 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

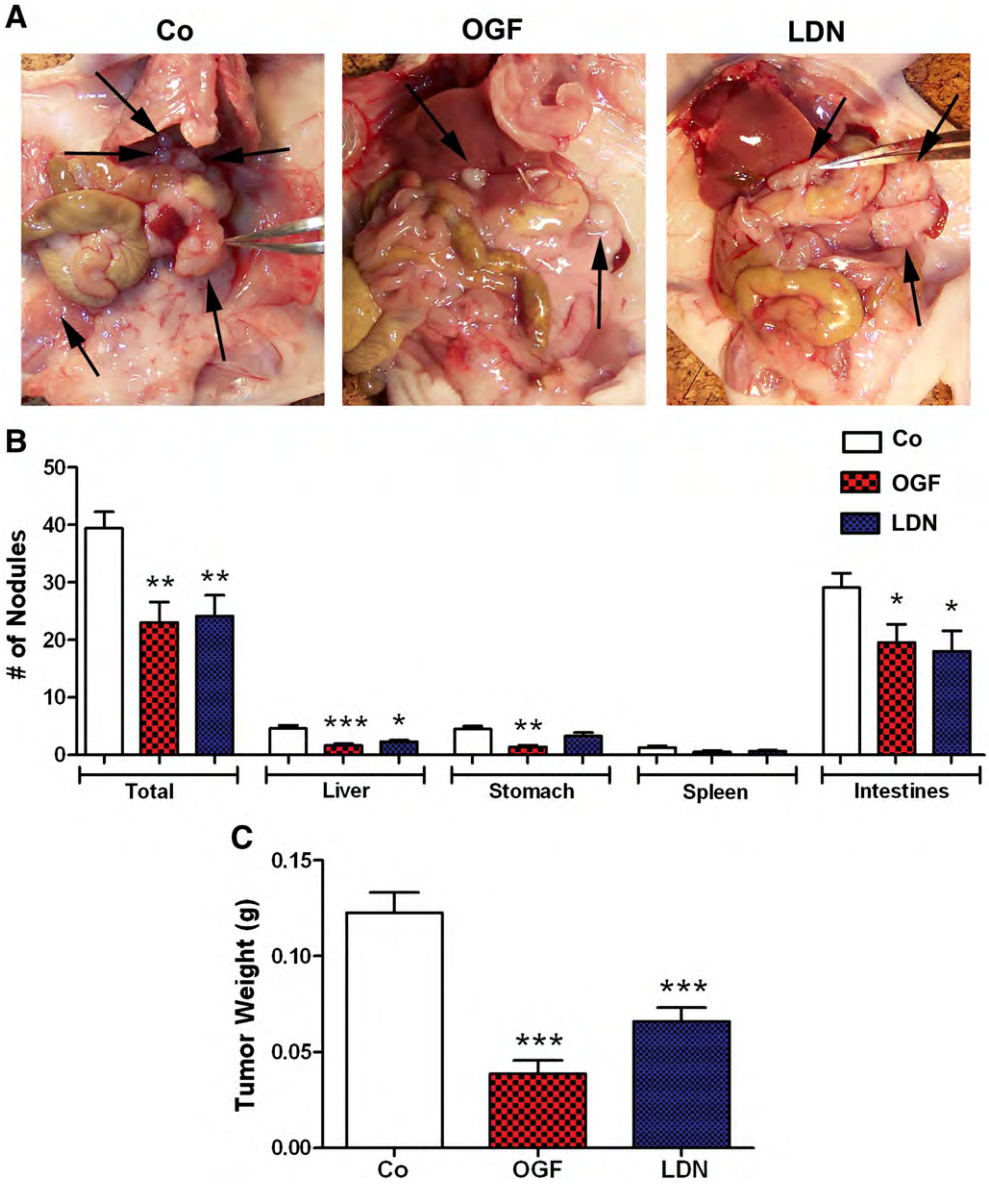

Fig. 1. Terminal tumor measurements at 40 days in mice with SKOV-3 intraperitoneal xenografts treated with OGF (10 mg/kg), LDN (0.1 mg/kg), or 0.2 ml of saline. (A) Representative images of the peritoneal cavity; arrows indicate tumors. (B) Number of tumor nodules. (C) Total tumor weight. Data represent means ± SE. Significantly different from the saline group (Co) at *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

of binding recorded in all groups (Fig. 3C). Binding capacity (B_{max}) was reduced in tumor tissue from mice receiving OGF (61%), but increased (90%) in animals administered with LDN, compared to preparations from saline injected control mice (Fig. 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 mm^2) in tumors from mice treated with OGF, LDN or saline (Figs. 4A and 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 (Figs. 4C and D). Similarly, blood vessel density in tumors was decreased in mice receiving OGF (89%) or LDN (73%), compared to saline administered controls (Figs. 4E and 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 related to 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

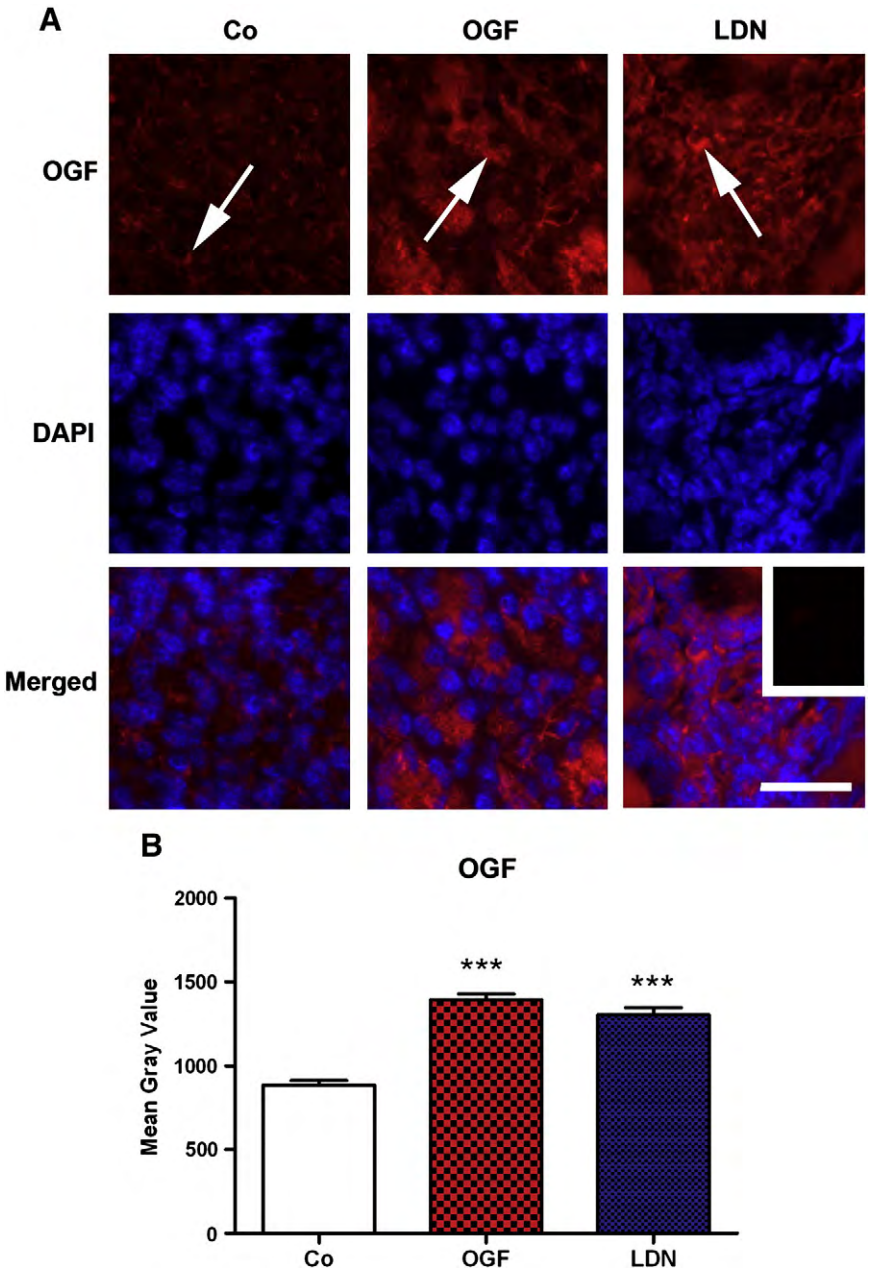

Fig. 2. The distribution and expression of OGF in tumors from mice with SKOV-3 intraperitoneal xenografts and treated with OGF, LDN, or saline for 40 days. (A) Photomicrographs of tumors stained with antibodies to OGF (1:200) were taken at the same exposure time. Rhodamine conjugated IgG (1:1000) served 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 $^{***}p<0.001$.

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 and 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, among a panel of natural and synthetic opioids, many specific for classic opioid receptors (μ, δ, and κ), OGF was the singular opioid peptide with growth inhibitory properties on ovarian cancer proliferation *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 G_1/S phase of the cell cycle without inducing apoptosis [5–8,33].

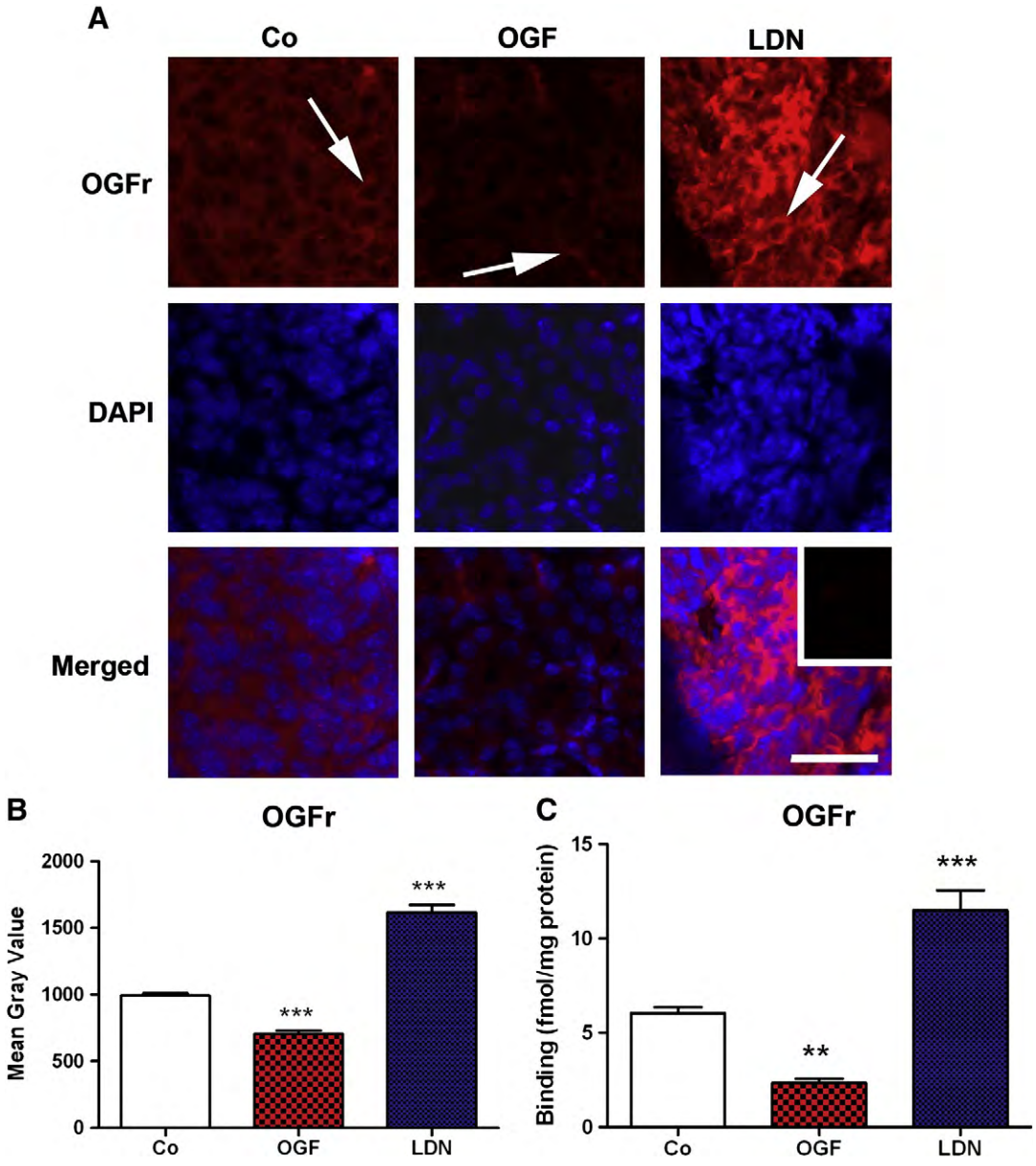

Fig. 3. The distribution and expression of OGFr in tumors from mice inoculated intraperitoneally with SKOV-3 cells and treated daily with OGF, LDN, or saline for 40 days. (A) Photomicrographs of tumors stained with antibodies to OGFr (1:200) were taken at the same exposure time. Rhodamine conjugated 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_{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 $^{***}p<0.001$.

The evidence in the present study reveals that upregulation of OGF–OGFr by exogenous OGF or LDN suppresses 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 G_1/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 blood 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 down-regulation 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

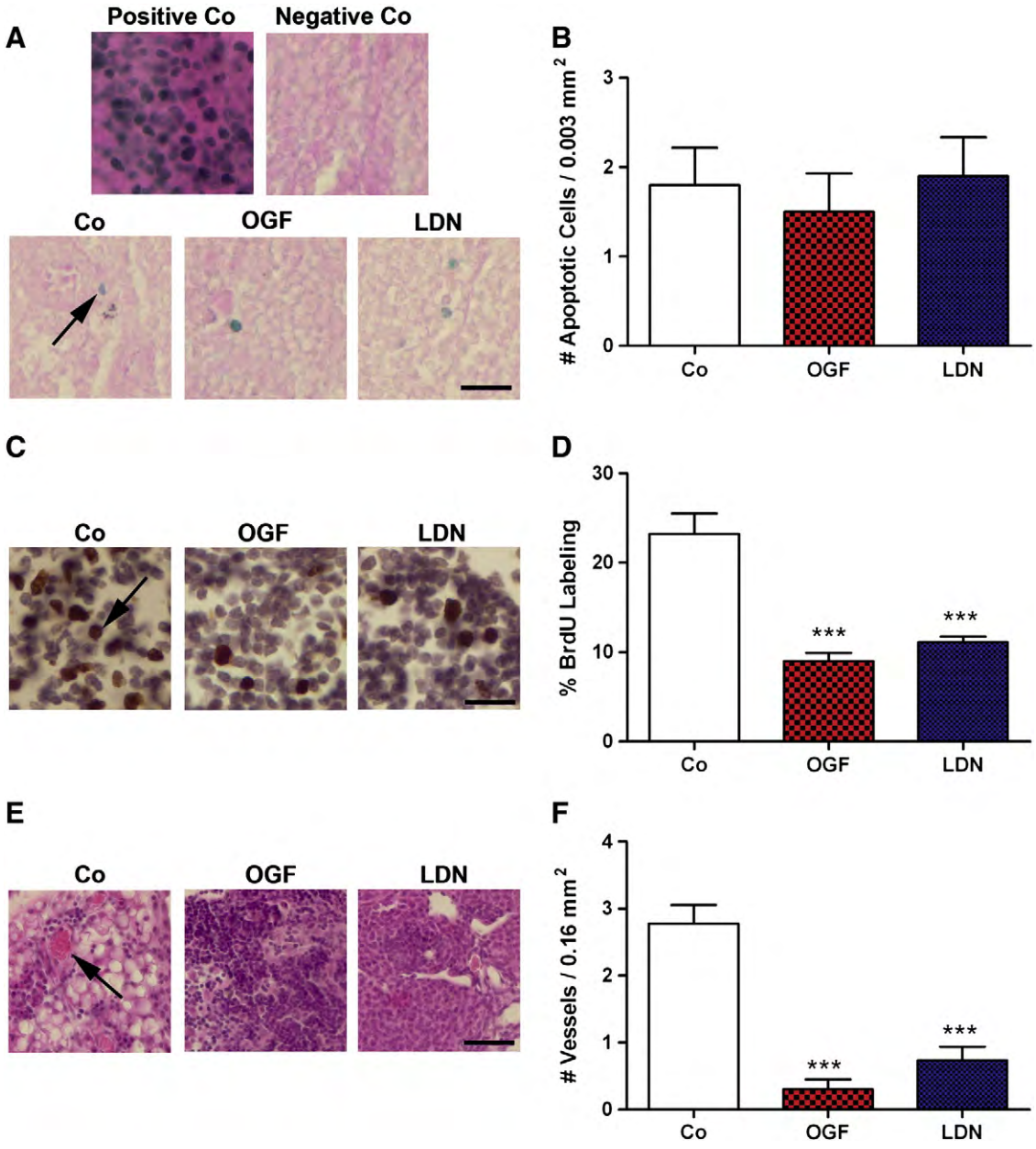

Fig. 4. Mechanism of tumor growth inhibition in mice with SKOV-3 intraperitoneal xenografts and treated daily with OGF, LDN, or saline for 40 days: Effects on apoptosis, DNA synthesis, and angiogenesis. (A) Photomicrographs of tumor tissue stained with TUNEL. Negative and positive controls were included according to the manufacturer's instruction. (B) Number of apoptotic cells per 0.003 mm^2. (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 mm^2. 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 ***$p<0.001$.

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 demonstrates that the OGF–OGFr axis is a determinant of ovarian cancer progression in a clinically relevant model of human ovarian cancer, and suggests that treatment with OGF or LDN merits consideration as a therapy for ovarian cancer.

Conflict of interest statement
No author has a financial conflict of interest in regard to this work.

Acknowledgments

This work was supported in part by the Paul K. and Anna E. Shockey Family Foundation and Bonnie and Ken Shockey. We thank Moshe Rogosnitzky for initial discussions about the efficacy of OGF in modulating human ovarian cancer.

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