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Expression of the opioid growth factor–opioid growth factor receptor axis in human ovarian cancer

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ABSTRACT

Objective. The opioid growth factor (OGF) and its receptor (OGFr), serve as inhibitory axis regulating cell proliferation in normal cells and cancer. We investigated the presence and relative expression of OGF and OGFr in normal human ovarian surface epithelial (HOSE) cells, benign ovarian cysts, and ovarian cancers.

Methods. Surgical samples of 16 patients with ovarian cancer and 27 patients with ovarian benign cysts were obtained intraoperatively. HOSE were collected by scraping the surface of normal ovaries of 10 post menopausal women undergoing hysterectomy and oophorectomy. Semiquantitative immunohistochemistry was used to assess the presence, distribution, and levels of OGF and OGFr. Receptor binding assays measured binding capacity and affinity of OGFr for radiolabeled OGF.

Results. OGF and OGFr were present in HOSE cells, ovarian cysts, and ovarian cancers. Compared to HOSE cells, OGF and OGFr protein levels were reduced 29% and 34% ($p<0.001$), respectively, in ovarian cysts, and decreased 58% and 48% ($p<0.001$), respectively, in ovarian cancers. Binding assays revealed 5.4 fold fewer OGFr binding sites in cancers than cysts ($p<0.05$). Levels of OGF and OGFr were comparable in primary, metastatic, or recurrent ovarian cancers.

Conclusion. We have shown that a native opioid pathway, the OGF–OGFr axis, is present in human ovarian cancer. Importantly, the expression of OGF and OGFr is diminished in human ovarian cancer. As OGF and OGFr normally function in maintaining cell proliferation, therapy to harness OGF/OGFr function could provide a useful biologic-based treatment for human ovarian cancer.

Introduction

About 22,000 women will be diagnosed with and 14,000 will die of epithelial ovarian cancer in the United States in 2010 [1]. Ovarian cancer is the most common cancer to present with advanced stage disease, 75% stages III–IV. From 1975 to 2005, there has been a statistically significant increase in the 5-year survival rate for ovarian cancer, from 37% to 46%, believed to be secondary to cytoreductive surgery and platinum/taxane chemotherapy [1]. Following cytoreductive surgery and platinum/taxane chemotherapy, over 80% of advanced ovarian cancers will be in remission. Unfortunately, 70% will recur and these women frequently will undergo secondary cytoreductive surgery and chemotherapy. Additional modalities are needed to prevent and treat recurrent disease, such as intraperitoneal chemotherapy, anti-vascular endothelial growth factor antibody, PARP (poly ADP-ribose polymerase) inhibitor, etc.

Endogenous opioid systems, consisting of opioid peptides and opioid receptors, have been reported to be associated with cancer [2,3]. Recently, we have identified a novel endogenous opioid system comprised of opioid growth factor (OGF, [Met^5]-enkephalin) and opioid growth factor receptor (OGFr) and documented the presence and function of the OGF–OGFr axis in human ovarian cancer cell lines (OVCAR-3, SKOV-3) [2–7]. OGF is a 573 molecular weight pentapeptide that is known to serve as a tonically active and direct inhibitory influence on cell proliferation in ovarian cancer cells in tissue culture that is dose-dependent, serum-independent, reversible, and receptor-mediated that was dependent on RNA and protein synthesis [2–7]. 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. The mechanism of OGF–OGFr action on DNA synthesis was related to the cyclin-dependent kinase inhibitory pathway because knockdown of p16 or p21 in OVCAR-3 cells, and p21 in SKOV-3 cells which lack p16, eliminated OGF's inhibitory effect on growth [2].

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Based on our experience with human ovarian cancer cell lines, we wished to investigate the presence and relative expression of OGF and OGFr in surgical specimens of normal human ovarian surface epithelial (HOSE) cells, benign cysts, and malignant cancers.

Materials and methods

Tissue samples

The specimens used in this study were obtained between July 2010 and January 2011 at The Milton S. Hershey Medical Center. All tissue samples were collected under an approved Institutional Review Board protocol. Surgical samples of ovarian cancer and ovarian cysts were obtained at the time of resection. Human ovarian surface epithelial (HOSE) cells were harvested, as previously described [8], by scraping the surface of normal ovaries of postmenopausal women undergoing uterine surgery for benign pathology and electing to have an oophorectomy. Tissue and cells were immediately placed in RPMI medium for transfer from the operating room to the laboratory. Within 45 min of collection, tumors and cysts were frozen in tris buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN) for binding analysis, or were flash frozen in isopentane chilled on dry ice for immunohistochemistry. HOSE cell suspensions were centrifuged, re-suspended in a small volume of RPMI medium, allowed to dry overnight on slides, and frozen at −80 °C. Samples were stored at −80 °C for up to 3 weeks. Laboratory investigators (RD, PM, IZ) were blinded to identification of the tissues (cyst vs cancer).

Semiquantitative immunohistochemistry

Immunohistochemistry and semiquantitative densimitry were utilized to evaluate the presence and relative levels of OGF and OGFr in tissues and cells following published procedures [4–7]. In brief, frozen tissue samples were sectioned on a cryostat at 10 μm, and transferred to gelatin coated slides. Cells and tissues were fixed, permeabilized, and stained with antibodies to OGF and OGFr (1:200) that were generated and characterized in our laboratory [3–5]. Sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Photomicrographs were taken with an IX-81 Olympus inverted microscope (Center Valley, PA) at the same exposure time with care not to photobleach samples. For tumors and cysts, staining intensity (mean gray value) was determined from a random sample of at least 5 fields/section and 3 sections/tissue, whereas for HOSE cells, mean gray value was assessed from at least 25 cells on 2 slides/sample. Controls consisted of specimens incubated with secondary antibodies only.

OGFr binding assays

Protein from ovarian tumors and cysts were prepared for binding assays as previously described [6]. Nuclear enriched protein from ovarian tumors and cysts (100–120 μg/tube) was incubated with custom synthesized [^{3}H]-[Met^5]-enkephalin (Perkin Elmer, Waltham, MA; 52.7 Ci/mmol) for 2 h at 22 °C with shaking. Saturation binding assays were conducted using isotope concentrations of 1 to 10 nM with samples assayed in duplicate. Incubations were terminated by filtering homogenates through Whatman GF/B fibers using a Brandel cell harvester (Gaithersburg, MD). Non specific binding was measured in the presence of unlabeled [Met^5]-enkephalin (10^{-5} M). Saturation isotherms calculating both binding capacity (B_{max}) and binding affinity (K_d) values were determined using GraphPad Prism software (GraphPad Prism, La Jolla, CA).

Statistical analysis

Age of subjects, body mass index (BMI), and mean gray values were analyzed using one way analysis of variance (ANOVA) with subsequent comparisons made using Newman–Keuls tests. B_{max} and K_d values associated with the binding assays were analyzed with unpaired two-tailed *t* tests. All analyses were performed using GraphPad Prism software.

Results

Subjects

Fifty three patients were included in this study (Table 1); in some cases, more than one sample of cancer was obtained from a patient (ovarian cancer and omental metastases). All ovarian cancers were advanced stage (3/4) and 88% were serous histology. A review of available patient demographics revealed that the mean age of the patients with ovarian cancer (58, range 41 to 83 yr), ovarian cysts (54, range 36 to 76 yr), and HOSE cells (59, range 46 to 79 yr) was similar ($p=0.50$). The BMIs of women providing cancer, cyst or HOSE cells were similar, with means ranging from 28 to 32 ($p=0.65$). More than 90% of patients in this study were Caucasian.

The presence and expression of OGF in patient samples

To evaluate OGF distribution and expression, semiquantitative immunohistochemistry was performed on HOSE cells, benign cysts, and malignant cancers. The location of OGF was similar in all groups, with immunoreactivity detected in the cytoplasm, and a speckling often noted in cell nuclei (Fig. 1A). Control samples processed with only secondary antibody showed no staining. OGF immunofluorescence (mean gray value − micrometers) was decreased 29% ($p<0.001$) and 58% ($p<0.001$) in cysts and cancers, respectively, compared to levels recorded in HOSE cells (Figs. 1B and C). OGF expression in cancer was reduced 41% ($p<0.05$) from that recorded in cysts. Expression of OGF in primary, metastases, and recurrent cancer was comparable (Figs. 1D and E).

The presence and expression of OGFr in patient samples

To evaluate the cellular distribution and expression of OGFr, semiquantitative immunohistochemistry was performed on patient samples of HOSE cells, benign cysts, and cancers. The location of OGFr was similar in all groups, with immunoreactivity detected in the cytoplasm, and often lightly scattered in cell nuclei (Fig. 2A). Control samples processed with only secondary antibody showed no staining. OGFr immunofluorescence (mean gray value) was decreased 34% ($p<0.001$) and 48% ($p<0.001$) in cysts and cancers, respectively, relative to those of HOSE cells (Figs. 2B and C). OGFr expression was reduced 22% ($p<0.001$) in ovarian cancers relative to levels recorded in cysts. Expression of OGFr in primary, metastases, and recurrent cancer was comparable (Figs. 2D and E).

Table 1
Demographics of patients assessed for expression of OGF and OGFr.

Characteristics	# of patients (%)	Age (mean±SE)	BMI (mean±SE)
Cancer	16 (30)	58±4	28±2
Primary	9 (17)	63±5	30±3
Metastases	4 (8)	54±5	26±2
Recurrent	6 (11)	54±6	27±2
Cysts	27 (51)	54±2	32±2
HOSE Cells	10 (19)	60±3	31±3

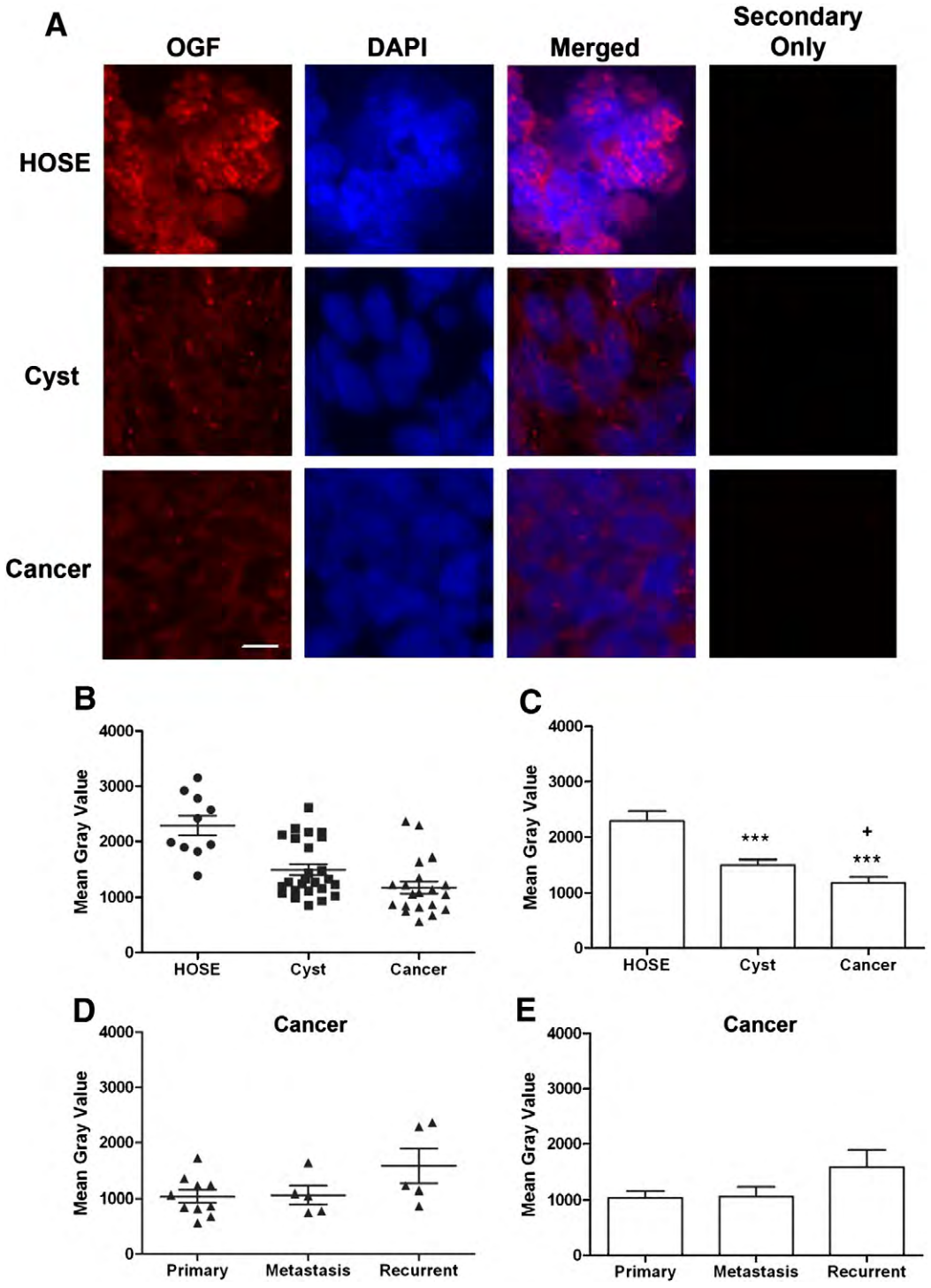

Fig. 1. The distribution and expression of OGF in HOSE cells, ovarian cysts, and ovarian cancers. (A)Photomicrographs of samples stained with antibodies to OGF (1:200). Rhodamine conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (secondary only) served as controls. Bar = 10 μm. (B, C) Scatter plot (B) and histogram (C) of semiquantitative measurement of OGF immunoreactivity (mean gray value). Data represent means + SE. Ovarian cancer significantly decreased compared to HOSE cells at ***$p<0.001$, and from ovarian cysts at +$p<0.05$. (D, E) Scatter plot (D) and histogram (E) of cancer samples in B and C separated into groups denoting primary, metastatic, or recurrent neoplasias. Data represent means + SE. No significant difference.

Receptor assays to assess binding characteristics of OGFr revealed specific and saturable binding to OGF in the nuclear fractions of benign cysts and cancer tissues, with a one site model of binding recorded in both groups (Figs. 3A, B). Values for binding capacity (B_{max}) were reduced 81% ($p<0.05$) in cancer tissues compared to that recorded in cysts. (Fig.3C). Binding affinity (K_d) ranged from 3.4 to 5.8 nM, and did not differ between cysts and cancers (Fig. 3D).

Discussion

Recently, we have identified a novel endogenous opioid system (OGF–OGFr) that inhibits cell proliferation, migration, angiogenesis, and tissue organization [2–7]. We have documented the presence of OGF–OGFr in human ovarian cancer cell lines (OVCAR-3, SKOV-3) [7]. In the present study we investigated the presence and relative

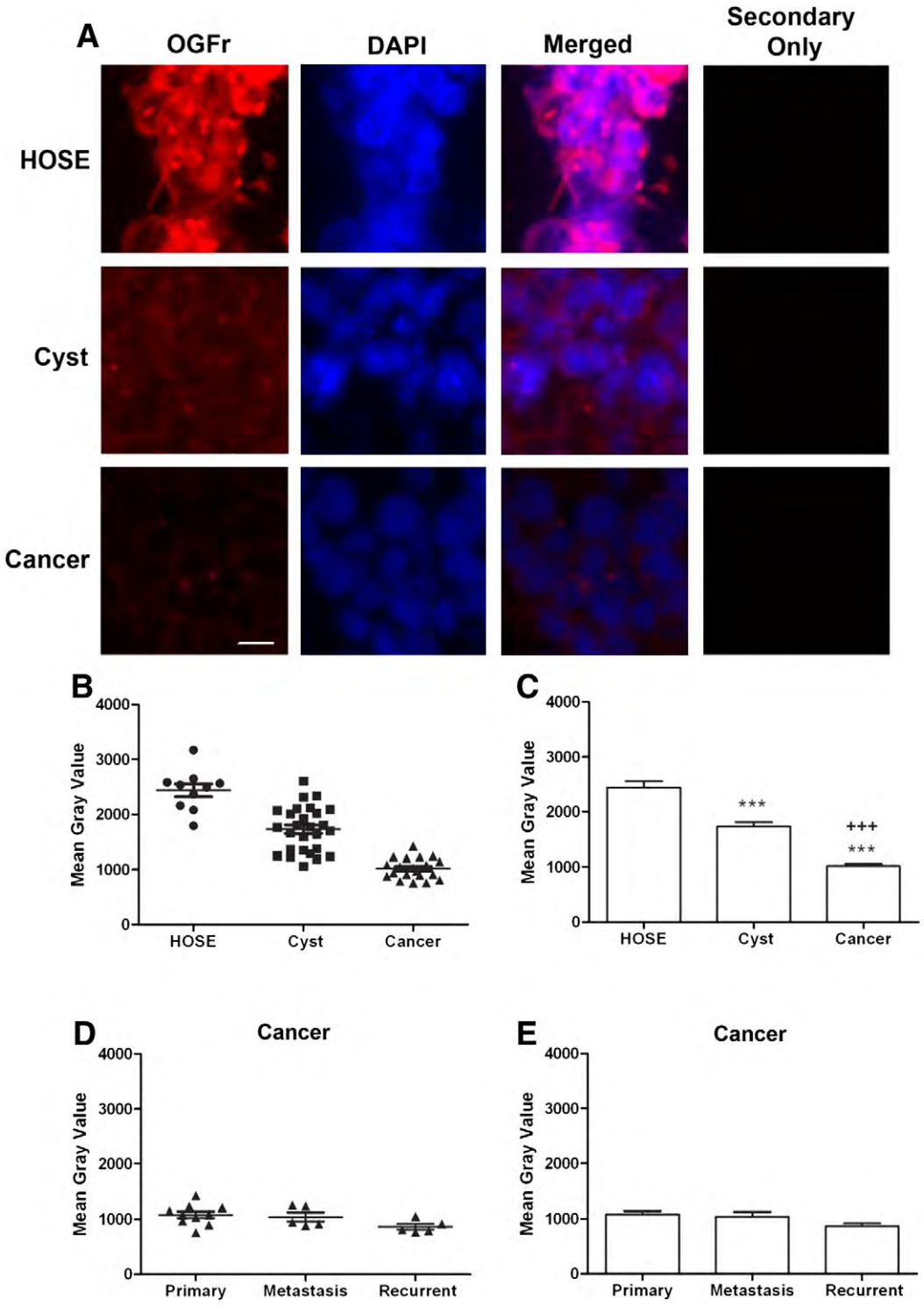

Fig. 2. The distribution and expression of OGFr in HOSE cells, ovarian cysts, and ovarian cancers. (A)Photomicrographs of samples stained with antibodies to OGFr (1:200). Rhodamine conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei were visualized with DAPI. Preparations incubated with secondary antibodies only (secondary only) served as controls. Bar = 10 μm. (B, C) Scatter plot (B) and histogram (C) of semiquantitative measurement of OGFr immunoreactivity (mean gray value). Ovarian cancer significantly decreased compared to HOSE cells at ***$p<0.001$, and ovarian cysts at +++$p<0.001$. (D, E) Scatter plot (D) and histogram (E) of cancer specimens in B and C separated into groups denoting primary, metastatic, or recurrent neoplasia. Data represent means + SE. No significant difference.

expression of OGF and OGFr in surgical specimens of normal human ovarian surface epithelial (HOSE) cells, benign ovarian cysts, and ovarian cancers. OGF and OGFr were present in HOSE cells, ovarian cysts, and ovarian cancers. Compared to HOSE cells, OGF and OGFr protein levels were significantly reduced 29% and 34% ($p<0.001$), respectively, in ovarian cysts, and significantly decreased 58% and 48% ($p<0.001$), respectively, in ovarian cancers. Binding assays revealed 5.4 fold fewer OGFr binding sites in cancers than cysts ($p<0.05$). To our knowledge, this is the original evaluation of OGF and OGFr in ovarian surgical specimens. Because OGF and OGFr are reduced in ovarian cysts compared to HOSE cells and OGF and OGFr are reduced in ovarian cancer compared to ovarian cysts, the OGF–OGFr endogenous opioid system may be associated with ovarian cancer carcinogenesis. Since all ovarian cancers were advanced stage and most were serous, we plan to evaluate OGF

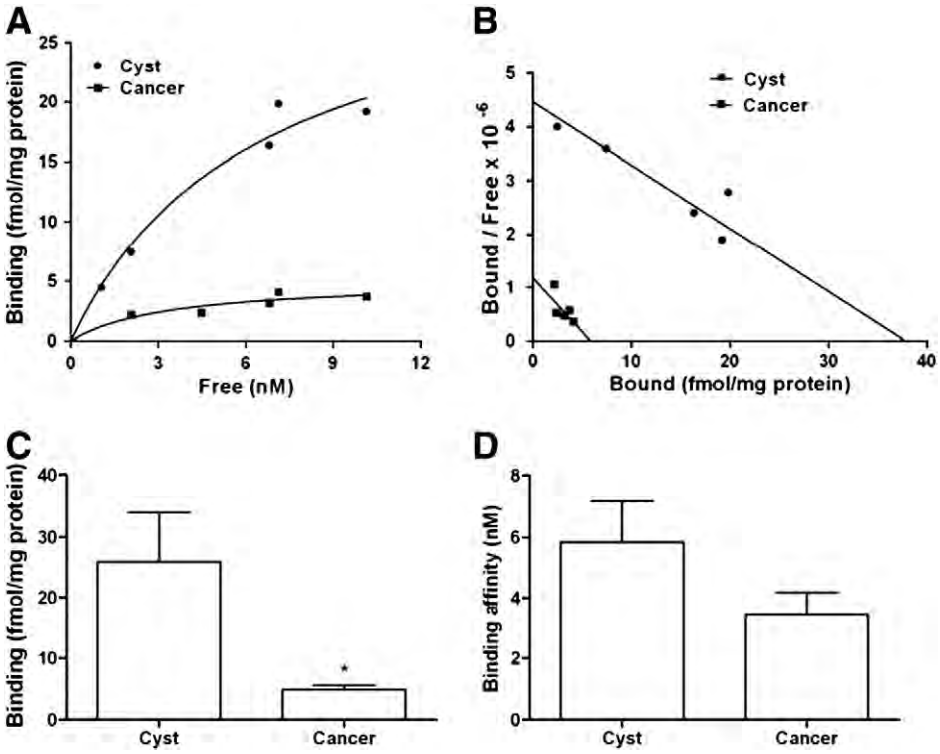

Fig. 3. Receptor binding assays of OGFr in benign cysts and malignant tumors. (A, B) Representative saturation isotherms (A) and Scatchard plots (B) calculating the binding capacity (B_{max}) and binding affinity (K_d) of OGFr in the nuclear fraction of tissue samples for radiolabeled OGF. (C) Histogram of binding capacity (B_{max}) values. (D) Histogram of binding affinity (K_d) values. Data represent means + SE. Ovarian cancer significantly decreased compared to ovarian cyst at $^*p<0.05$.

and OGFr in early stage cancer, borderline tumors and various histologic subtypes.

Since OGF and OGFr normally function in regulating cell proliferation, therapy to restore normal OGF/OGFr function could provide a novel therapy for human ovarian cancer. Using an i.p. and s.c. mouse model of human ovarian cancer (SKOV-3) [10], we have developed two means of modulation of the OGF–OGFr axis to decreased cancer growth: injection of OGF and the use of a low dose of the opioid antagonist naltrexone (LDN) [9,12]. In the case of OGF, exogenous OGF appears to upregulate the OGF–OGFr pathway resulting in decreased cancer growth [9]. With respect to LDN, opioid receptor blockade causes a compensatory increase in OGF and OGFr. By administering a short (4–6 h/day) opioid receptor blockade each day, the remaining 18–20 h/day when the LDN is no longer present allows for the interaction of the elevated opioids and opioid receptors, producing a suppression of cell proliferation [11,12]. The combination of OGF or LDN and cisplatin demonstrated additive inhibitory effects such as reduced tumor size and substantial decreases in angiogenesis and DNA synthesis [9,12]. Moreover, OGF had a protective action by reducing the toxic effects (e.g., weight loss) of chemotherapy [9]. In phase 1 trials we have shown the safety of OGF and naltrexone (LDN) in pancreatic cancer and Crohn's disease. In a phase 1 trial of OGF in 16 patients with pancreatic cancer, a MTD of 250 μg iv weekly was established [13]. Side effects were minimal and there was no bone marrow suppression, gastrointestinal disturbance, or renal/hepatic toxicity. Two of 16 patients (13%) had a partial response. In a phase 1 trial of LDN in 17 patients with Crohn's, a MTD of 4.5 mg po daily was established [14]. Side effects were minimal (except for sleep disturbance in 41%) and there was no bone marrow suppression, gastrointestinal disturbance, or renal or hepatic toxicity. Remission was achieved in 67% of patients.

In conclusion, we have identified the presence of the OGF–OGFr endogenous opioid system in human ovarian cancer specimens. Secondary to its high recurrence rate, ultimate chemoresistance and subsequent lethality despite conventional cytotoxic therapies, novel approaches are needed for the treatment of advanced epithelial ovarian cancer. Opioid growth factor and its receptor represent an attractive target. Previous in-vitro and in-vivo studies have shown the OGF/OGFr axis to be susceptible to receptor agonist intervention with resultant inhibition of cell proliferation, angiogenesis and tumor growth. We have further substantiated the role of OGF–OGFr in ovarian tumorigenesis by demonstrating protein expression of OGF and OGFr in ovarian cancer specimens. Furthermore, phase I trials have established OGF as relatively non-toxic with a lack of severe side effects. These findings warrant further exploration in a clinical trial setting. We are currently developing protocols to incorporate OGF and naltrexone, in combination with standard adjuvant chemotherapies, for the treatment of recurrent epithelial ovarian cancer.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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