

Opioid growth factor receptor is unaltered with the progression of human pancreatic and colon cancers

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Abstract. Opioid growth factor (OGF) is an endogenous opioid peptide ([Met⁵]-enkephalin) that interacts with the OGF receptor (OGFr). OGF serves as a constitutively expressed and tonically active negative growth factor in neoplasia, and the OGF-OGFr axis contributes to the maintenance of an equilibrium in cell replication by targeting cyclin-dependent inhibitory kinase pathways. In a previous study, OGFr binding activity was found to decrease in concert with progression of human squamous cell carcinoma of the head and neck (SCCHN). To investigate the relationship of OGFr to advancement of human pancreatic and colon cancers, tumor cells were transplanted into nude mice, and small, medium, and large neoplasias were assessed for OGFr number and affinity by receptor binding analysis, and for gene expression of OGFr mRNA by Northern blot analysis. In addition, OGF levels were monitored in plasma. OGFr binding affinity and capacity, as well as transcriptional activity of OGFr, were not influenced by the size or state of differentiation of pancreatic or colon tumors. Plasma levels of OGF were 3.5- to 7.9-fold less in animals with pancreatic or colon cancers than in nude mice not receiving xenografts, and no differences in OGF values were recorded between small and large tumors. These data on human pancreatic and colon cancers, along with information in earlier studies on SCCHN, indicate that alterations in the OGF receptor are dependent on tumor type and that the integrity of the OGF-OGFr axis insofar as tumorigenesis needs to be evaluated for each type of neoplasm. This information will be relevant in the design of therapeutic modalities, the diagnosis and prognosis of neoplasia, as well as understanding of the processes and mechanisms of carcinogenesis.

Introduction

Opioid growth factor (OGF) and its receptor (OGFr) play a role in the regulation of growth of human cancer cells in tissue culture (1-5) and tumors in nude mice (6-9). OGF serves as a tonically active endogenous pentapeptide that inhibits cell proliferation in a receptor-mediated manner (1-5,10). OGF action is unrelated to apoptosis/necrosis (11) or differentiation (12), and displays anchorage-independent properties (13). The mechanism of action of OGF appears to be the up-regulation of cyclin-dependent inhibitory kinases in order to delay the G₀/G₁ phase of the cell cycle (14). OGF and OGFr have been demonstrated by immunohistochemistry, radioimmunoassay, and/or receptor binding to be present in a variety of human and animal tumors and cancer cell lines (1-5,8,15-19). Immunoelectron microscopic studies (20,21) reveal that OGFr resides on the outer nuclear envelope, where OGF interacts with OGFr, and the OGF-OGFr complex translocates between the cytoplasm and nucleus at the nuclear pore by interaction with karyopherin β . Thus, signal transduction for cell proliferation appears to involve an OGF-OGFr complex that interfaces with chromatin in the nucleus.

Clinical studies have documented specific and saturable binding of OGF in surgical specimens of pancreatic cancers (16), colon cancers (15), and squamous cell carcinoma of the head and neck (SCCHN) (17-19), indicating the presence of the OGF receptor. McLaughlin and colleagues (19) showed a 9-fold decrease in OGFr binding activity, and a 5-fold decrease in OGFr protein, from tumor tissue surgically removed from patients with SCCHN compared to normal epithelium; transcription of OGFr was comparable between SCCHN and normal samples. To fully examine the relationship of tumor progression and levels of OGFr, McLaughlin and Zagon (22) established an animal model of small, medium, and large SCCHN xenografts. These investigators found that OGFr binding capacity was 3- to 7-fold lower in large tumors of SCCHN than in small tumors, with no change in OGFr mRNA noted between small and large tumors. These results suggest that, at least for SCCHN, there is a progressive decrease in OGFr with advancement of neoplasia. Importantly, the results of this study in an animal model of SCCHN corroborate clinical findings.

The present study was designed to address the relationship of OGFr to tumor progression in other types of cancer known to have OGF receptor. Using 3 different pancreatic cancer cell

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lines, and 2 different colon cancer cell lines that represented poorly and well-differentiated cancers, subcutaneous tumors were collected from nude mice as 'small', 'medium', and 'large' in size (i.e., diameter, weight, volume). Receptor binding assays were performed to determine binding affinity (K_d) and capacity (B_{max}). Gene expression of OGF_r was quantitated by densitometric analysis of Northern blots. Finally, to investigate the response of OGF levels in the plasma of animals with small, medium, and large tumors, radioimmunoassays were performed.

Materials and methods

Cancer cell lines. Three human pancreatic cancer cell lines (MIA PaCa-2, BxPC-3, and Capan-2) and 2 human colon adenocarcinoma cell lines (HT-29 and HCT 116) were used in this study. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). The MIA PaCa-2 cell line was derived from an undifferentiated epithelial carcinoma occurring in the body and tail of the pancreas in a 65-year-old man (23). The BxPC-3 cell line originated from an adenocarcinoma of the body of the pancreas from a 61-year-old female, and was characterized histologically as a moderately well to poorly differentiated adenocarcinoma (24). The Capan-2 cell line was derived by Fogh and Trempe from a well-differentiated adenocarcinoma in the head of the pancreas in a 56-year-old Caucasian male (26). The HT-29 cell line was derived from a well-differentiated primary colon adenocarcinoma that was established from a 44-year-old Caucasian female (26), whereas the HCT 116 cell line was derived from a poorly differentiated primary adenocarcinoma obtained from an adult male (27).

MIA PaCa-2 cells were maintained in Dulbecco's MEM (modified) media, HT-29 and HCT 116 were grown in McCoy's 5A media, and BxPC-3 and Capan-2 cells were cultured in RPMI. All media were supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 units/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin). Cell cultures were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. The cells were harvested with 0.05% trypsin/0.53 mM EDTA, centrifuged, and counted with a hemacytometer. Cell viability was determined by trypan blue staining.

Tumor cell implantation, tumor growth, and tissue collection. Male 4-to 5-week-old BALB/c-nu/nu nude mice, purchased from Harlan Laboratories (Indianapolis, IN), were housed in pathogen-free isolators in the Department of Comparative Medicine at the Penn State University College of Medicine. All procedures were approved by the IACUC committee of the Penn State University College of Medicine and conformed to the guidelines established by NIH. The mice were allowed at least 5 days to acclimate prior to beginning experimentation.

Nude mice received a single subcutaneous injection into the right scapular region of the following: 2×10^6 MIA PaCa-2, 10^6 BxPC-3, 3×10^6 Capan-2, 5×10^6 HT-29, and 10^5 HCT 116 cells per mouse. For each cell line, at least 12 mice were utilized. Cell number was titrated to that which produced 100% tumors within approximately 2 weeks of cell inoculation.

Mice were observed daily for the initial appearance of tumors. Tumors were measured twice weekly using calipers. Tumor volume was calculated using the following formula: $w^2 \times l \times \pi/6$, where the length is the longest dimension, and width is the dimension perpendicular to length (28). Tumors were harvested as small, medium, or large and corresponded to dimensions of approximately 10, 20, or 40 mm, respectively, in one diameter.

When tumors were of the appropriate size, mice were euthanized by an overdose of sodium pentobarbital, and whole blood from some mice was collected into heparinized tubes from the heart. Plasma was separated from whole blood and stored at -70°C. Tumors were measured, removed and weighed. Tumor tissue was flash frozen in liquid nitrogen for receptor binding assays, frozen in isopentane chilled on dry ice for immunohistochemistry, or placed in guanidine isothiocyanate buffer (GIT) and stored at -70°C for RNA assays. Tissues were processed within 2 weeks of collection.

Receptor binding assays. Dependent on tumor size, 1-3 tumors were sampled for each assay, and at least 3 binding assays were performed on each size tumor. Tumor tissues were removed, washed free of blood and connective tissue, immediately frozen in liquid nitrogen, and assessed following modifications of the procedures published previously (8,9,17,29). Tumors were homogenized in 0.32 M sucrose in Tris-buffer containing protease inhibitors (= Tris/all buffer). Nuclear protein homogenates were diluted with Tris/all buffer to the appropriate protein concentration and incubated at room temperature (22°C) for 10 min to remove endogenous opioid peptides. Aliquots (0.95 ml) of protein were incubated with 50 μ l of [³H]-[Met⁵]-enkephalin (custom synthesized by Perkin-Elmer Life and Analytical Sciences, Boston, MA) and appropriate blockers. Non-specific binding was determined in the presence of 100 nM unlabeled [Met⁵]-enkephalin. Duplicate tubes of homogenates were assayed for each concentration. Protein concentrations were determined by the Bio-Rad method with gamma globulin as a standard.

Northern blot analysis of OGF_r gene expression. OGF_r gene expression was assessed for MIA PaCa-2 and HCT 116 tumors; at least 3 independent Northern blots/size/tumor, with each lane representing RNA from an individual tumor, were utilized. Total RNA was isolated from tumors by standard procedures (19). RNA was precipitated with ethanol and quantitated by UV spectrometry. Inasmuch as possible, equal amounts of total RNA were subjected to electrophoresis on 1% agarose-2.5% formaldehyde gels. Northern blot analysis of OGF_r gene expression was performed as described earlier (19). To control for differences in the amount of RNA loaded, as well as the integrity of the RNA, blots were stripped and reprobed with [³²P]-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Autoradiograms were analyzed for relative OD using a laser scanning densitometer (Molecular Dynamics/Amersham, Sunnyvale, CA) with PDQUEST software. The OD values for radiolabeled OGF_r in individual blots were normalized to the OD values for GAPDH.

Measurement of OGF in plasma. OGF levels in plasma of mice with small, medium, and large MIA PaCa-2, BxPC-3,

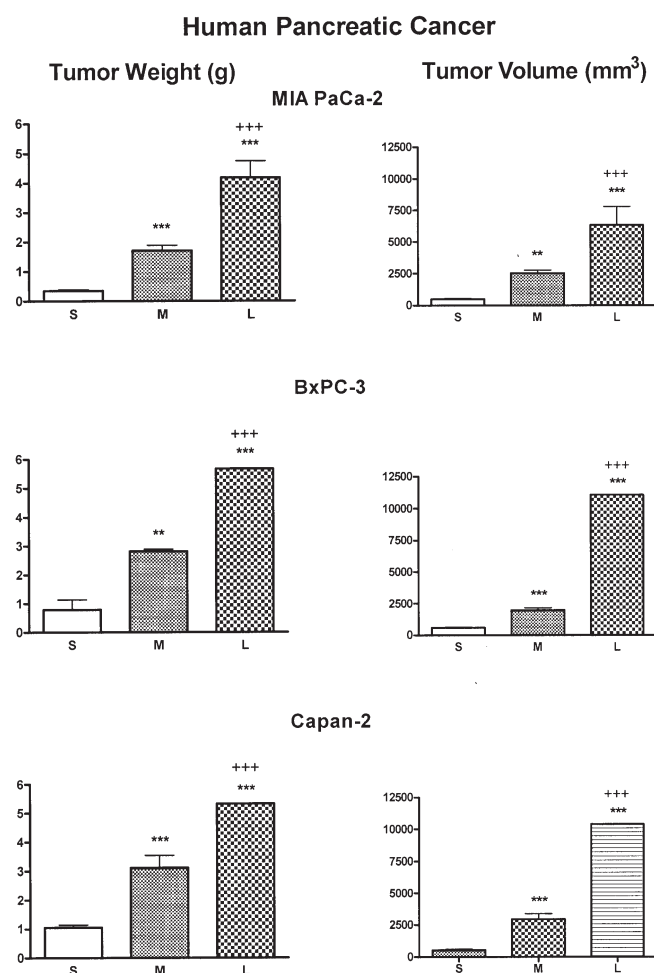


Figure 1. Tumor weight and tumor volume of small, medium, and large tumors derived from MIA PaCa-2, BxPC-3, and Capan-2 human pancreatic cancer cell lines. Data represent means \pm SEM for at least 10 tumors for each size. Significantly different from small tumors at ** $p<0.01$ and *** $p<0.001$, and from medium tumors at *** $p<0.001$.

and Capan-2 human pancreatic, and HT-29 colon adenocarcinoma, tumors were measured by standard radioimmunoassay procedures using a kit from Peninsula Laboratories (Belmont, CA).

Data analysis. Tumor weights and volumes, B_{max} and K_d values, and plasma OGF values were analyzed by analysis of variance (ANOVA); subsequent planned comparisons were made with Newman-Keuls tests. Data from binding assays were analyzed by GraphPad Prism 4.0 software; binding affinities and capacities, as well as Scatchard plots, were generated by the computer program.

Results

Body weight. Athymic nude mice weighed approximately 22 g at the beginning of experimentation. No significant differences were noted in the body weights of mice bearing small, medium, or large tumors within a tumor type, although mice with large tumors weighed slightly more, presumably due to the weight of the tumor. Thus, at the time of termination, mice with MIA PaCa-2, BxPC-3, and Capan-2 tumors had

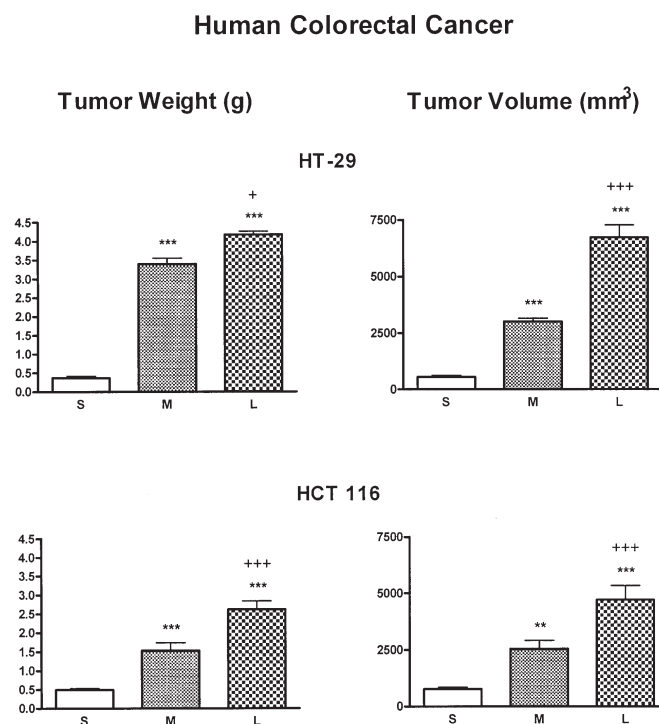


Figure 2. Tumor weight and tumor volume of small, medium, and large tumors derived from HT-29 and HCT 116 human colon cancer cell lines. Data represent means \pm SEM for at least 10 tumors for each size. Significantly different from small tumors at ** $p<0.01$ and *** $p<0.001$, and from medium tumors at * $p<0.05$ () and *** $p<0.001$.

body weights of 28.0 ± 1.2 , 31.8 ± 1.6 , and 33.4 ± 0.1 g, respectively. Animals with HT-29 and HCT 116 tumors had body weights of 28 ± 1.2 and 24.6 ± 0.7 g, respectively.

Tumor volume and tumor weight. Weights and volumes of small, medium, and large tumors in mice receiving MIA PaCa-2, BxPC-3, or Capan-2 cells differed significantly (Fig. 1). For example, in the BxPC-3 group, the weights of medium and large tumors were 3.6- and 7.2-fold, respectively, greater than that of the small tumors, and the large tumors were 2.0-fold greater than those in the medium size group. Likewise, in the BxPC-3 group, tumor volumes for medium and large tumors were 3.3- and 18-fold, respectively, greater than that of small size tumors, and the large size tumors were 5.6-fold greater than those in the medium size group.

Weights and volumes of small, medium, and large tumors in mice receiving human colon cancer cell lines HT-29 or HCT 116 differed significantly (Fig. 2). For example, in the HCT 116 group, the weights of medium and large tumors were 3.0- and 5.2-fold, respectively, greater than that of the small size tumors, and the large tumors were 73% greater than those in the medium size group. Likewise, in the HCT 116 group, tumor volumes for medium and large tumors were 3.3- and 6.0-fold, respectively, greater than that of small tumors, and the large tumors were 1.8-fold greater than those in the medium size group.

OGFr receptor binding analyses. Radiolabeled OGF binding assays revealed specific and saturable binding of the ligand to OGFr, with binding curves that indicated a single site

Table I. Binding affinity of [³H]-[Met⁵]-enkephalin in nuclear homogenates of human pancreatic and colon cancer cells xenografted into nude mice.

Cancer type Cell line	Tumor size		
	Small	Medium	Large
Pancreatic cancer			
MIA PaCa-2	1.5±0.4	1.3±0.4	2.2±0.6
BxPC-3	1.8±0.4	1.9±0.7	1.6±0.3
Capan-2	1.7±0.1	2.2±0.6	1.0±0.3
Colon adenocarcinoma			
HT-29	1.6±0.5	1.0±0.3	1.5±0.8
HCT 116	12.9±0.7	7.3±2.1	1.4±0.3

Values represent means ± SEM for at least 3 assays. Data did not differ between any tumor size within each cancer cell line.

for each of the 3 pancreatic and 2 colon cancer cell lines examined.

Binding affinity. Binding affinity (K_d) did not differ between assays for small, medium, and large tumors derived by inoculation with any of the pancreatic or colon cancer cell lines (Table I).

Binding capacity. Values for binding capacity (B_{max}) for small, medium, and large tumors derived by inoculation with any of the pancreatic or colon cancer cell lines revealed no significant differences (Fig. 3).

OGF_r gene expression. Quantitative analysis of OGF_r gene expression as measured by RNA isolated from small, medium, or large MIA PaCa-2 pancreatic tumors or HCT 116 colon adenocarcinomas demonstrated similar expression profiles of OGF_r normalized to GAPDH independent of tumor size (Fig. 4). Total RNA from MIA PaCa-2 or HCT 116 cells in culture was also analyzed for OGF_r gene expression. Densitometric analysis of OGF_r mRNA normalized to GAPDH revealed no differences in gene expression between cancer cells *in vitro* and small, medium, or large tumors (data not shown).

OGF levels in plasma. Serum levels of OGF in nude mice were assessed in blood collected at the time of death. OGF levels in mice with small or large tumors derived from MIA PaCa-2, Capan-2 or BxPC-3 pancreatic cancer cells did not differ (Fig. 5). No differences in serum OGF levels were detected in mice with small or large HT-29 colon tumors (Fig. 5). In comparison to plasma levels of OGF in nude mice never subjected to cancer cells, mice bearing pancreatic or colon tumors had significantly 3.4- to 7.9-fold less OGF; this difference in plasma OGF levels between mice with tumors, and those not inoculated with cancer cells, was $p < 0.001$.

OGF_r Binding Capacity

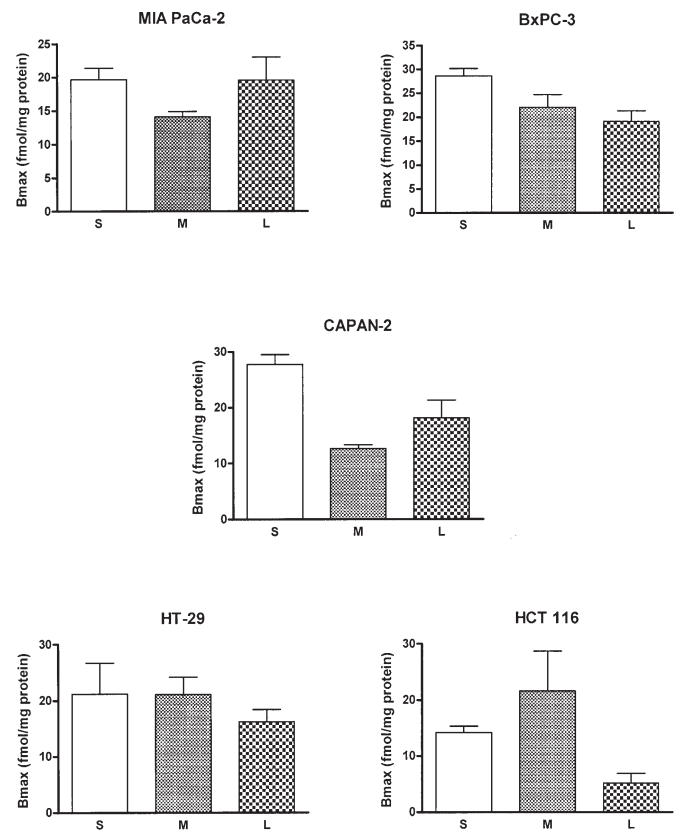


Figure 3. Binding capacity (B_{max}) values of OGF_r for small, medium, and large tumors derived from MIA PaCa-2, BxPC-3, Capan-2, HT-29, and HCT 116 cancer cell lines. Histograms represent means ± SEM for at least 3 saturation binding isotherms. Data did not differ between any tumor size within each cancer cell line.

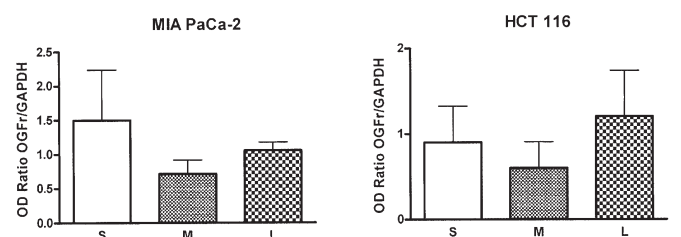


Figure 4. OGF_r gene expression in specimens of small, medium, and large tumors derived from inoculation of nude mice with MIA PaCa-2 human pancreatic adenocarcinoma cells or HCT 116 human colon cancer cells. Northern blot analysis of total RNA isolated from tumors was probed with human OGF_r cDNA; filters were stripped of isotope and reprobed with GAPDH, a constitutively expressed mRNA. The histogram presents optical density ratios. Values represent means ± SEM. There were no statistical differences between tumors of various sizes in gene expression.

Discussion

Data generated in this study demonstrate that OGF_r binding affinity and binding capacity, as well as transcriptional activity of OGF_r, are not influenced by the size and, hence, progression of human pancreatic or colon cancer. The basis for this finding was our strategy to compare OGF receptor

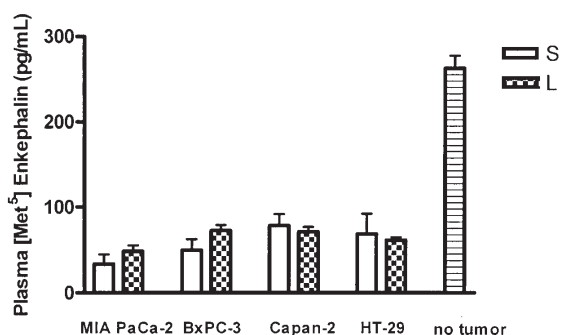


Figure 5. OGF levels in plasma of mice with small or large MIA PaCa-2, BxPC-3, Capan-2, and HT-29 tumors; plasma of athymic nude mice never inoculated with cancer cells was also evaluated (= no tumor). Values represent means \pm SEM. The plasma levels of OGF for all cancers (small and large) differed significantly from mice with no tumors.

binding kinetics (K_d , B_{max}) and OGF mRNA in tumors that were documented to be substantially different in diameter, weight, and volume. In addition, these results were consistent across cell lines within a cancer, and for two distinct cancer types. The use of pancreatic and colon cancer cell lines, representative of poorly to well-differentiated neoplasias (23-27), demonstrated that the magnitude of OGF binding activity was not dependent on the state of differentiation. Thus, for the first time, our results with xenografts of human neoplasias show that progression of pancreatic and colon cancers are not associated with alteration in the OGF receptor.

The absence of changes in OGF binding activity observed in this study contrasts with results documented by our lab (9-22) showing the relationship of OGF to the progression of SCCHN. Previous reports by McLaughlin and Zagon (22) revealed that progression of SCCHN is accompanied by a decrease in the number of OGF receptors, and no change in binding affinity of OGF or gene expression of OGF mRNA. Comparison of the present observations, where no changes in OGF receptors were recorded for pancreatic and colon cancers, with those of SCCHN suggests that not all cancer types are affected similarly with respect to the OGF-OGFr axis.

In the present study, a systematic examination of the relationship of plasma levels of OGF in pancreatic and colon cancers was undertaken. The results show that mice with either cancer have markedly subnormal levels of OGF, regardless of the size of their tumor. Plasma levels of OGF have been reported previously for SCCHN (9-29), colon cancer (30), and pancreatic adenocarcinoma (7). When comparisons to non-tumor animals were made, no changes in plasma OGF levels were observed with SCCHN (9-29), but animals with BxPC-3 pancreatic tumors had 4.4-fold more OGF in plasma than normal (non-tumor injected) mice (7). It is unclear why the data in the present study differ from those reported previously (7,9,29). However, differences in radioimmunoassay kits and/or extraction procedures may account for this discrepancy. Data showing reductions in plasma OGF, along with a lack of change in OGF receptor number, with regard to cancer progression suggest that the events related to this peptide and receptor have to be considered separately.

The functional implications of a decrease in OGF levels toward cancer growth for pancreatic and colon neoplasia need

to be considered. OGF is a tonically active inhibitory growth factor that interacts with OGFr to maintain a homeostatic equilibrium of cell replication. A decrease in OGF levels in the plasma of animals bearing pancreatic or colon cancer would suggest that there is a reduction in the amount of peptide required to optimally regulate DNA synthesis, with the result being an enhancement in tumor cell number. Indeed, antibody blockade of OGF activity in pancreatic cells, for example, markedly elevates the number of tumor cells from those treated with vehicle or non-immune IgG (3). Moreover, addition of exogenous OGF, either under *in vitro* (3,29) or *in vivo* (7,29) conditions, appears to compensate for the reduction in endogenous OGF.

The OGF-OGFr axis is important in the regulation of homeostatic cell proliferation, and the clinical implications of alterations in this system are of interest. The reduction in OGF levels in nude mice bearing human pancreatic and colon cancers would suggest that there is a defect in one element (i.e. OGF) of the OGF-OGFr axis. The net effect of a reduction in plasma OGF levels would be attenuation of the inhibitory action of this peptide, and an increase in cell replication and disease progression. As a corollary, administration of exogenous OGF would be expected to compensate for the loss of endogenous OGF because the receptor remains intact. Our studies support this hypothesis both in the laboratory (1,3,6,7,29) and clinically (30). Administration of exogenous OGF to patients with pancreatic cancer has successfully passed Phase I trials (30), and this study indicated that patients with unresectable pancreatic cancer receiving OGF had a marked increase in survival compared to historical controls.

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