Opioid growth factor-opioid growth factor receptor axis is a physiological determinant of cell proliferation in diverse human cancers

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Zagon IS, Donahue RN, McLaughlin PJ. Opioid growth factor-opioid growth factor receptor axis is a physiological determinant of cell proliferation in diverse human cancers. Am J Physiol Regul Integr Comp Physiol 297: R1154–R1161, 2009.—The opioid growth factor (OGF) regulates cell proliferation of human cancer cells through the cyclin-dependent kinase inhibitory pathway, with mediation of this action by the OGF receptor (OGFr). The ubiquity of the OGF-OGFr axis in human cancer is unknown. We used 31 human cancer cell lines, representative of more than 90% of neoplasias occurring in humans, and found that OGF and OGFr were detected in the cytoplasm and nucleus by immunohistochemistry. The addition of OGF to cultures depressed cell number up to 41%, whereas naltrexone (NTX) increased cell proliferation by up to 44%, a total of 85% in the modulating capacity for the OGF-OGFr axis. Neutralization of OGF by specific antibodies led to a marked increase in cell number. Knockdown of OGFr by OGFr-siRNA resulted in a significant increase in the number of cells, even in the face of the addition of exogenous OGF. The cultures to which NTX was added and subjected to OGFr-siRNA were similar to those with OGFr-siRNA alone. The OGF-OGFr axis, a physiological determinant of cell-proliferative activity, is a ubiquitous feature of human cancer cells. The identification of this native biological system in neoplasia may be important in understanding the pathophysiology of neoplasia, and in designing treatment modalities that utilize the body’s own chemistry.

DYSREGULATION OF CELLULAR proliferation is an integral part of the cancer phenotype (33, 44). One native biological regulator of DNA and cell replication in cancer and normal cells is the opioid growth factor (OGF) and its receptor, OGF receptor (OGFr) (10, 13, 14, 73). OGF, chemically termed [Met5]-enkephalin, is a pentapeptide that is constitutively expressed, autocrine produced, and secreted to inhibit the proliferation of neoplastic and normal cells (73). The action of OGF is tonic, stereospecific, noncytotoxic, and nonapoptotic inducing, not associated with differentiative, migratory, invasive, or adhensive processes, independent of serum, anchorage-independent, reliant on RNA and protein synthesis, and occurring at physiologically relevant concentrations (14, 63–65, 67, 73). OGFr consists of a 677-amino acid protein (71, 73), has a chromosomal location at 20q13.3 (71), and contains nuclear localization signals that are determinants of cell-proliferative properties (12). The OGF-OGFr axis delays the G1/S interface of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways (10, 11, 13). Attenuation of the OGF-OGFr axis in cancer cells by 1) disruption of OGF-OGFr interfacing through continuous exposure to opioid antagonists (e.g., naltrexone, NTX) (5, 14, 36, 42, 58, 70, 2) neutralization of OGF by antibodies to the peptide (14, 36), or 3) a decrease in OGFr by antisense cDNA or siRNA for OGFr (10, 11, 13, 14, 56, 74), stimulates cell proliferation. An increase in OGF-OGFr activity in cancer cells by 1) the addition of exogenous OGF (5, 10, 13, 14, 36, 42, 58, 70); 2) treatment with imidazoquinoline compounds, such as imiquimod and resiquimod (56); or 3) transfection of sense cDNA for OGFr (41, 72), depresses cell proliferation.

Studies with tumor transplantation of human colon and pancreatic adenocarcinomas, as well as squamous cell carcinoma of the head and neck, in immunocompromised mice treated with OGF has demonstrated decreases in tumor incidence, delays in tumor onset and progression, and/or extended survival (25, 38, 39, 57, 59, 60). Moreover, OGF and OGFr have been identified in several types of human tumors obtained at surgery (19, 24, 31, 37, 40, 66, 69). Clinical studies have successfully identified a nontoxic dosage of OGF (49), and Phase II clinical trials using OGF treatment are under way in several cancers (J. Smith and D. Goldenberg, personal communication). Given the presence and importance of the OGF-OGFr axis in human tumors, we raised the question of the ubiquity of this biological system in human cancer. Using 31 human cancer cell lines representing systems and sites of disease responsible for more than 90% of new cases of cancer, as well as those responsible for deaths (26), we tested in this study four lines of evidence related to the ubiquity and functional importance of the OGF-OGFr axis: 1) the presence of OGF and OGFr, 2) the effects of OGF on cell proliferation, 3) the repercussions on cell replication when OGF was depleted in cultures by antibody neutralization, and 4) the consequences of knocking down OGFr with siRNA as to cell growth. The present investigation documents that the OGF-OGFr axis is a physiological determinant of cell-proliferative activity and is a ubiquitous feature of human cancer cells.

MATERIALS AND METHODS

Cell culture. Human cancer cell lines, CAL 27 (18), MIA PaCa-2 (55), BxPC-3 (52), HT-29 (16), HCT 116 (7), SK-OV-3 (16), OVCAR-3 (20), H226 (48), A549 (17), DU 145 (51), PC-3 (27), SK-HEP-1 (16), Hep G2 (29), HT-1080 (47), SK-ES-1 (9), SW 1088 (15), U-87 MG (3), U251 (54), SK-N-SH (4), MDA-MB-231 (9), MCF7 (8), K-562 (32), AGS (2), U266 (45), MES-SA (21), and Caki-2 (16), were obtained from the American Type Culture Collection (Manassas, VA). Flo-1 (50) was a gift from Dr. D. Beer (University of Michigan), SCC-1 (30) was a gift from Dr. T. E. Carey (University of Michigan Cancer Research Laboratory), UACC903 (53) and 1205 LU (23) were gifts from Dr. G. Robertson (Penn State...
Fig. 1. Opioid growth factor receptor (OGFr) is present, and gene and protein expression is reduced by transfection with OGFr siRNA, in SCC-1, MIA PaCa-2, and KAT-18 cells. A: Northern blot analysis demonstrating the specificity and level of OGFr mRNA knockdown. Log phase cells were transfected for 24 h with either scrambled siRNA or OGFr siRNA and examined 48 h later. Data (percent of OGFr/GAPDH ratio) are expressed as means ± SE for two blots from independent experiments. Significantly different from nontransfected cultures at \( P < 0.01 \) (**), or \( P < 0.001 \) (***)

B: Photomicrographs of log-phase cells stained with an antibody to OGFr demonstrating that immunoreactivity was associated with the cytoplasm, along with a speckling of stain in cell nuclei. Immunostaining was not detected in cells incubated with secondary antibodies only (inset). Photomicrographs of cells stained with OGFr were taken at the same exposure time. Semiquantitative measurements showed a subnormal OGFr staining intensity (mean gray value) in cultures transfected with OGFr siRNA. Data represent mean ± SE for 100 cells/treatment sampled from 3 coverslips/treatment. Significantly different from nontransfected cells at \( P < 0.001 \) (***). Scale bar = 10 \( \mu \)m.
RESULTS

OGFr is present in human cancer cells. OGFr mRNA was detected in RNA extracted from log phase SCC-1, MIA PaCa-2, and KAT-18 cultures (Fig. 1A). Immunoreactive OGFr was localized to the cytoplasm of SCC-1, MIA PaCa-2, and KAT-18 cells, with a speckling of immunoreactivity noted
in the nucleus (Fig. 1B). No staining was recorded in specimens processed with secondary antibody only.

Silencing of OGFr in human cancer cells blocks the inhibitory action of endogenous and exogenous OGF and the stimulatory action of NTX. The requirement of OGFr for OGF’s inhibitory action on cell proliferation was evaluated at the molecular level using siRNA technology. SCC-1, MiaPaCa-2, and KAT-18 cells transfected with OGFr siRNA had reductions of 22%, 51%, and 76%, respectively, in OGFr mRNA levels relative to untransfected cells (Fig. 1A). However, cells exposed to scrambled siRNA were comparable in OGFr mRNA levels to untransfected cells. Semiquantitative immunohistochemistry of OGFr revealed that SCC-1, MiaPaCa-2, and KAT-18 cells transfected with OGFr siRNA had reductions of ~30% in OGFr protein levels in contrast to untransfected cells (Fig. 1B). Cancer cells transfected with OGFr siRNA had 38–54% more cells than in untransfected cultures (Fig. 2). The addition of exogenous 10^{-6} M OGF or NTX had no inhibitory or stimulatory effects, respectively, on cells transfected with OGFr siRNA compared with cultures transfected with OGFr siRNA and treated with sterile water.

OGF is present in human cancer cells. Immunoreactive OGF was localized to the cytoplasm of SCC-1, MiaPaCa-2, and KAT-18 cells, and a speckling of immunoreactivity was noted in the nucleus (Fig. 3A). No staining was recorded in specimens processed with secondary antibody only.

The endogenous opioid specific for growth inhibition of human cancer cells is OGF. The specificity of endogenous OGF’s inhibitory action was investigated by neutralizing native OGF with a polyclonal antibody. SCC-1, MiaPaCa-2, and KAT-18 cultures exposed to the OGF antibody had 31% to 42% more cells than control cultures treated with sterile water or those receiving preimmune serum (Fig. 3B).

The OGF-OGFr axis is present and functions in a wide variety of human cancer cells. The ubiquity of OGF’s inhibitory action in human cancer cells was examined in 28 additional cell lines, representing 20 cancer types. After 72 h of treatment with OGF, cells were reduced in number by 18% to

Fig. 3. The presence and function of endogenous OGF in SCC-1, MIA PaCa-2, and KAT-18 cancer cells. A: photomicrographs of log-phase cells stained with antibodies (1:100) to OGF; rhodamine-conjugated IgG (1:1,000) served as the secondary antibody. Immunostaining was associated with the cytoplasm, and a speckling of stain was noted in cell nuclei. Immunostaining was not detected in cells incubated with secondary antibodies only (inset). Scale bar = 10 μm. B: neutralization of endogenous OGF. Cultures were treated with a specific antibody to OGF (Co172), preimmune serum (IgG), or with exogenous OGF (10^{-6} M); media, antibodies, and peptides were replaced daily. Cell number was measured at 72 h. Data are expressed as means ± SE for at least 2 aliquots/well from at least 2 wells/group. Significantly different from respective controls at *P < 0.001 (***).
41%, from sterile water-treated controls, while exposure to NTX increased cell number from 8% to 44%, relative to cells treated with sterile water (Fig. 4). Cultures transfected with OGFr siRNA had 19% to 61% more cells than cultures that were not transfected (Fig. 5). The addition of exogenous OGF or NTX had no inhibitory or stimulatory effects, respectively, on any of the 29 additional cell lines examined that were transfected with OGFr-siRNA compared with cultures treated with OGFr-siRNA and sterile water. Although all cell lines responded to OGFr-siRNA or NTX treatment, the magnitude of difference between the effects of OGFr-siRNA and NTX ranged from 3 to 42%; these differences were significant ($P < 0.05$ or greater) for 18 cell lines.

**DISCUSSION**

This study shows for the first time that a biological system, OGF-OGFr, is present and functions as a determinant of cell proliferation in a wide variety of human cancer cells that represent over 90% of the major sites and systems affected by this disease (26). This included one or two neoplastic cell lines for the oral cavity/pharynx, breast, leukemia, myeloma, soft tissues, skin, digestive (esophagus, stomach, liver, pancreas, colon/rectum), respiratory, genital, urinary, endocrine, and brain and nervous systems. Four lines of evidence support this conclusion. First, both OGF and OGFr were detected in the cytoplasm and the nucleus, an observation consistent with previous reports in human cancer, as well as normal cells (5, 14, 36, 42, 58, 70). The OGF-OGFr axis is known to undergo nucleocytoplasmic transport (12, 68), and the present data are consonant with transport of a peptide-receptor complex between the cytoplasm and the nucleus. Second, OGFr is known to be an inhibitor of DNA synthesis and subsequent cell replication (5, 10, 11, 13, 14, 36, 42, 58, 70). The addition of exogenous OGF to the cultured cells resulted in a marked depression in cell number. Third, OGFr is known to be constitutively expressed by cells and to exhibit activity through a feedback loop with OGFr (73). In the present studies, neutralization of OGF using a specific antibody produced a marked increase in cell number. These data suggest that the tonic action of the autocrine produced and secreted OGF with its receptor, OGFr, is interrupted and thereby removes an important biological axis that maintains the pace of cell proliferation. Fourth, OGFr is known to be the mediator of OGF activity (73). Experiments performed herein using siRNA to knock down OGFr yielded cell cultures that had a notable increase in cell number, indicating that interference of peptide-receptor interaction compromises the functioning of an important determinant of the rate of cell proliferation. Thus, our study makes the novel finding that a native biological system, which serves as a primary regulator of DNA synthesis and cell replication appears to be an ubiquitous feature of human cancer cells.

The finding of a functional OGF-OGFr system in the 31 human cell lines confirms and extends previous observations in some of these cell lines (5, 14, 36, 42, 58, 70). Moreover, earlier studies also have reported the presence and/or functioning of this peptide-receptor interfacing in human cell lines not utilized herein. This includes human cell lines of renal cancer: A498, SN12C, ACHN, and Caki-1 (5), pancreatic adenocarcinoma: Capan-1, Capan-2, and PANC-1 (70), colon adenocarcinoma: SW480, WiDr, and COLO 205 (58), melanoma: G361 (71), promyelocytic leukemia: HL-60 (71), neuroblastoma: SK-N-AS (71), ovarian: CAOV-3 and SW626 (14), and squamous cell carcinoma of the head and neck (SCCHN): SCC-4, SCC-9, and SCC-25 (36). Thus, a total of 49 human cell lines have been documented to be regulated in cell-proliferative activities by the OGF-OGFr axis, extending further the concept of ubiquity of this biological system in most (if not all) human cancers.

An additional observation relevant to the ubiquity of the OGF-OGFr axis emanates from the inclusion of the effects of the opioid antagonist, NTX, in these studies. NTX is known to block the interfacing of OGF and OGFr, resulting in a release of the tonically active inhibitory influence of OGF on cell replication, and a consequent increase in cell number. Indeed, all of the cell lines used were found to be markedly elevated when NTX was added to the cultures. This is a consistent principle of OGF-OGFr action, as reported previously (5, 14, 36, 42, 58, 70). NTX is a general opioid antagonist for opioid receptors, and this raises the question of whether NTX acts only through the OGF-OGFr pathway, or also by way of other opioid-opioid receptor systems in regard to growth. In the present investigation, the silencing of OGFr increased cell number. Moreover, all cell lines except for one transfected with OGFr-siRNA demonstrated increased cell number relative to cultures exposed to NTX alone. It should be noted that there was a wide range of response to treatment with OGFr-siRNA or NTX. This could be the result of differences in basal OGFr levels, transfection efficiencies of OGFr-siRNA, and/or levels of signaling molecules involved in proliferative pathways (e.g., p16, p21). If other opioid-receptor systems in addition to OGF-OGFr were involved in growth, NTX would be expected to increase cell number beyond the levels measured with OGFr knockdown. Because this was not observed, even when NTX was added to cultures treated with OGFr-siRNA, the data would suggest that only the OGF-OGFr axis is the opioid pathway regulating cell proliferation in these cancer cells. This conclusion supports the earlier observations of Cheng and colleagues for pancreatic (10) and SCCHN (13) cancers, as well as Donahue and coworkers for ovarian cancer (14), in which the extent of the cell increase generated by OGFr-siRNA was comparable or greater than NTX alone. These results also 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 (68). 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 (73). Thus, NTX appears to have recognition of OGFr in these cancer cells but may not provide a complete blockade of OGF-OGFr interfacing.

Other opioid peptides tested in culture with a variety of human (ovarian, SCCHN, pancreatic, colorectal) and animal cancer cells, many of which are selective for classical opioid receptors (μ, δ, κ), have not been found to alter growth (14, 36, 58, 70), indicating the specificity of the OGF-OGFr axis in cell proliferation. It also should be noted that some investigators have found that certain opioid agonists can depress the number of cancer cells, but do so in either a “partially reversible” or irreversible fashion by opioid antagonists (22, 28, 43, 46). These findings argue for a nonopioid receptor and support the present results showing that a general opioid antagonist does not elevate cell proliferation greater than that with OGFr-siRNA because no other opioid-receptor system is involved. These data are consonant with the finding that NTX’s capacity to disrupt opioid-regulated growth may be directed to a singular opioid system: OGF-OGFr.

The mechanism of OGF action on cell proliferation has been reported for three types of cancer: pancreatic, SCCHN, and ovarian (10, 13, 14). In each case, OGF appears to delay the cell cycle at the G1-S interface by an upregulation in CKIs p16 and/or p21, which depresses Cdk4/Cdk2 kinase activity and the phosphorylation of Rb protein, leading to a decrease in DNA synthesis and subsequent cell proliferation. Whether the CKI pathway is a common denominator in the mechanism of OGF-OGFr for all of the human cancers now shown to be regulated by this peptide-receptor axis, remains to be elucidated.

The identification and function of the OGF-OGFr axis as a ubiquitous feature in human cancer cells in the present study was confined to cell lines. It may be argued that this is not the case in human tumor cells growing in vivo. However, tumor transplantation studies with pancreatic and colon adenocarcinoma, as well as SCCHN, have documented that exogenous administration of OGF can decrease tumor incidence, delay tumor appearance, and retard growth of these neoplasias (25, 34, 35, 38, 39, 57, 59–61). Moreover, OGF and OGFr have been recorded in human tumors transplanted into nude mice; surgical samples of pancreatic, colon, and thyroid cancer; and SCCHN, using immunohistochemistry and/or receptor-binding techniques (19, 24, 31, 69). Thus, any reservations in translating our findings in tissue culture to the human condition are minimized by these studies. However, whether all cancers respond to OGF in tumor transplantation studies, or whether OGF and OGFr are resident in all tumors from patients, remains to be investigated.

The clinical implications of these findings are twofold. First, we have identified a common biological pathway in the regulation of
cancer cell proliferation, a hallmark of neoplasia (33, 44). Thus, further information about the pathways of this peptide-receptor system may be used in understanding the etiology and pathogenesis of cancer. For example, the onset and/or progression of some cancers may be related to defects in OGF and/or OGFr, which would, in turn, attenuate the inhibitory action on cell proliferation, thereby exacerbating tumorigenesis. Second, knowing that the OGF-OGFr pathway is present and a determinant of proliferation in cancer cells, could be used in designing treatment strategies. For example, one could take advantage of upregulating either the peptide (e.g., exogenous OGF) and/or receptor (e.g., gene delivery, iniquimod) to enhance anticancer activity. OGF has been successfully documented to be safe for administration in humans (49), and Phase II trials with OGF in pancreatic cancer patients, as well as individuals with SCCHN, are under way (J. Smith and D. Goldenberg, personal communication). Additionally, Phase I trials using OGF in combination with chemotherapy for treatment of pancreatic cancer (J. Smith, personal communication), along with the use of OGF for hepatocellular carcinoma (E. Kimchi and K. Staveley O’Carroll, personal communication) are being conducted. Thus, our finding that the OGF-OGFr axis is a ubiquitous feature of human cancer provides a basis for discovery of treatment modalities.

Perspectives and Significance

These data are the first to reveal that the OGF-OGFr axis, a native biological system, is present and functions as a tonically active negative regulator of cell proliferation in cancers representative of more than 90% of neoplasias occurring in humans. The findings are not only important in our understanding of the biology of cancer but can be used in expanding strategies for diagnosis of these neoplasias. Additionally, this novel information may be of importance in designing treatments that take advantage of the body’s own chemical processes to restore normal homeostasis in pathophysiological conditions.

GRANTS

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REFERENCES
