Prevention and delay in progression of human squamous cell carcinoma of the head and neck in nude mice by stable overexpression of the opioid growth factor receptor

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Abstract. This study examined overexpression of the opioid growth factor receptor (OGFr) in squamous cell carcinoma of the head and neck and phenotypic repercussions on tumorigenicity. Tumors from 3 SCC-1 cell lines (OGFr-9, OGFr-18, OGFr-22) stably transfected with OGFr cDNA (OGFr-1) had 2.5- to 3.7-fold more OGFr than empty vector (EV) or wild-type (WT) neoplasias. No differences in OGFr number were detected between tumors of EV and WT animals. Only 16 and 28% of the mice in the OGFr-18 and OGFr-22 groups, respectively, receiving 2 million tumor cells had a measurable tumor on day 12 compared to 70% of the EV group; 25% of the OGFr-22 animals given 5 million cells expressed a tumor relative to the EV group (100%). Latencies for tumor appearance were extended by 25 and 80% for animals in the OGFr-18 and OGFr-22 groups, respectively, compared to EV animals given 2 million cells, and were lengthened by 2-fold in OGFr-22 animals injected with 5 million cells. Tumor weight of all animals overexpressing OGFr were 48-67% of EV mice, and the number of cells undergoing DNA synthesis in these tumors with amplified OGFr was reduced 46-65% of the EV group. Tumor volumes of OGFr-9 animals inoculated with 2 million cells and followed for over 7 weeks were reduced 36-70% from the WT group on days 31-54. Tumor weights on day 54 for the OGFr-9 group were 2.6-fold less than those for the WT animals. These data support OGFr gene function as a regulator of cell proliferation that impacts on tumorigenic expression of SCCHN, and suggests that molecular and pharmacological manipulation of OGFr may prevent or delay human head and neck squamous cell cancers.

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Introduction

Cancer of the head and neck (including oral cavity, pharynx, and larynx) accounts for 3% of new cases and 2% of cancer deaths annually in the USA (1). Worldwide, these neoplasias affect over 600,000 individuals each year (2,3), and rank as 10th in global cancer incidence and 8th in global cancer deaths (3). Greater than 90% of head and neck cancers are squamous cell carcinomas (SCCHN) (4). There is a greater than 50% chance of recurrence of advanced-stage SCCHN within 2 years of intervention, with a median survival of 6 months and a 1-year survival of 20% (4,5). The survival rate for SCCHN of the oral cavity has improved slightly (5%) in the past several decades for White (~62%), but not African (~41%), Americans, and the rate for SCCHN of the larynx (~64%) has remained the same for all races (1). This poor survival rate is not only due to a high frequency of recurrence and second primary tumors, but also to the late stage of diagnosis, despite multimodality therapy including surgery, radiation therapy, and chemotherapy (6,7). The pain, disfigurement, and physical disabilities that often arise from the disease and its treatment have serious effects on the quality of life of survivors (8).

The opioid growth factor (OGF), chemically termed [Met^5]-enkephalin, is an endogenous opioid peptide that is an important regulator of the progression of human SCCHN (9-21). OGF is a constitutively expressed native opioid that is autocrine produced and secreted, and interacts with the OGF receptor (OGFr) to inhibit the growth of SCCHN cells *in vitro* and in tumor xenografts (10,12,16-24). The action of OGF is tonic, stereospecific, reversible, non-cytotoxic and non-apoptotic inducing, not associated with differentiative, migratory, invasive, or adhesive processes, independent of serum, anchorage-independent, and occurs at physiologically relevant concentrations in a wide variety of SCCHN cancers including poorly- and well-differentiated human cell lines (10,23-26). The only opioid peptide, natural or synthetic, that influences the growth of SCCHN is OGF (10), and this peptide has been localized by immunohistochemistry to SCCHN cells in culture, xenografts, and surgical specimens (9,10,20). The action of this opioid in these neoplasias is targeted to DNA synthesis (10,12,21,22) and is directed toward the p16 cyclin-dependent inhibitory kinase (CKI) pathway (22) which delays cells from transiting G_1 to the S-phases of the cell cycle. Molecular evidence for involvement of CKI in OGF action comes from knockdown experiments with CKIs that

eliminates the effects of OGF as an inhibitory peptide (22). Attenuation of OGF in SCCHN cells through: i) disruption of OGF-OGFr interfacing by way of continuous exposure to opioid antagonists (e.g., NTX) (10,12,22); or ii) a decrease in OGF by antibodies to the peptide (10) stimulates cell proliferation. An increase of OGF in SCCHN cells by addition of exogenous OGF depresses cell proliferation (10,12,21,22).

The gene for human OGFr is at least 9 kb in length, and consists of seven exons and six introns (27,28). OGFr is a 677 amino acid protein that includes 7 imperfect repeats of 20 amino acids each and 3 nuclear localization signals (27,28), and has an apparent mass of 62 kDa (27,28). The chromosomal location of the human OGFr is 20q13.3 (28). Although OGFr has pharmacological properties (e.g., recognizes opioids, naloxone reversibility, stereospecificity) of classical opioid receptors, there is no homology of OGFr in terms of nucleotides or amino acids (28). OGFr has been detected by immunohistochemistry in SCCHN cells in culture, xenografts, and surgical specimens (9,10,19-21). Using immunoelectron microscopy, OGFr has been observed on the outer nuclear envelope and in the nucleus and perinuclear cytoplasm of rat tongue epithelium (29). OGFr in surgical samples of SCCHN is markedly decreased from normal epithelium, and is also reduced in the margins of SCCHN tumors that are normal by pathological observation (15). Moreover, McLaughlin and Zagon (19), utilizing different size nude mouse tumors from xenografts of human SCCHN, reported a progressive decrease in translation/posttranslation of OGFr protein, but not transcriptional levels of the OGFr gene. An increase in OGFr by: i) treatment with imidazoquinoline compounds such as imiquimod and resiquimod (30); or ii) transfection of sense cDNA for OGFr (31,32) depressed cell proliferation. A decrease in OGFr by: i) antisense RNA (27,28), or ii) knockdown by siRNA for OGFr (22,30) results in an elevation in cell proliferative activity.

In a previous study using tissue culture, overexpression of OGFr at the molecular level in human SCCHN cells resulted in marked decreases in DNA synthesis and cell proliferation (21). The present investigation was directed towards addressing whether amplification of OGFr in human SCCHN cancer cells transplanted into mice contributes to phenotypic changes in tumorigenicity. The results show for the first time that overexpression of OGFr at the molecular level prevents or delays tumor appearance, retards tumor progression, and depresses DNA synthesis of these cancers. Thus, a biologic approach to upregulate, OGFr, under *in vivo* conditions may provide a novel target that inhibits SCCHN suggesting that molecular and pharmacological strategies could be utilized for the treatment of this deadly neoplasia.

Materials and methods

Cell lines. Human SCCHN cell line, UM-SCC-1 (SCC-1), obtained from the University of Michigan Cancer Research Laboratory (Dr Thomas E. Carey, Director), was maintained in culture with Dulbecco's modified Eagle's medium (DMEM) (Penn State Media Center), containing 10% heat-inactivated fetal bovine serum, 3.7 g/l $NaHCO_3$ and antibiotics (10^5 U/l penicillin, 100 μg/ml streptomycin). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Gene constructs and transfections. A previously described plasmid, pcDNA3.1 + huOGFr (21), was used for obtaining stable expression of human OGFr transgene in human SCCHN cells. Clonal cell lines OGFr-9, OGFr-18, OGFr-22, and cells transfected with empty vector (EV) were expanded and characterized as reported earlier (21). These cells, as well as wild-type (WT) SCC-1 cells, were analyzed by receptor binding assays to determine OGFr receptor number prior to inoculation into nude mice.

Tumor burden studies in nude mice. Four-week-old male BALB/c athymic nude mice were purchased from Harlan Laboratories (Indianapolis, IN), and acclimated to flexible-film isolators for one week prior to tumor cell inoculation. Two inoculums ($2x10^6$, $5x10^6$) of WT, EV, OGFr-9, OGF-18, and OGFr-22 cells were injected subcutaneously into the right scapula region (~0.2 ml/mouse) of nude (nu/nu) mice.

Mice were observed daily for initial appearance of tumors, and subsequently measured in 2 dimensions using vernier calipers (16,17,19,20). Tumor volume was calculated as $l \times w^2 \times \pi/6$ where w is the shorter dimension (33). Depending on the experiment, mice were euthanized and tumors removed for analysis.

OGF receptor binding analyses. Cells in culture, as well tumor tissues, were assayed for OGFr using custom synthesized [^{3}H]-[Met^5]-enkephalin (Perkin-Elmer, Boston, MA). Binding assays for cells and tumor tissues followed the procedures by McLaughlin *et al* (11). Non-specific binding was measured in the presence of unlabeled [Met^5]-enkephalin. Saturation binding isotherms were generated using GraphPad Prism software; K_d and B_{max} values were provided by computer software.

Immunohistochemistry. To examine for the presence of OGFr, SCCHN tumors were excised and frozen in isopentane chilled on dry ice at the time of euthanasia. Tumors were sectioned and stained with ammonium sulfate purified anti-OGFr-IgG (1:200; I0028) (34) diluted in Sorenson's phosphate buffer with 1% normal goat serum and 0.1% Triton X-100 for 18 h at 4˚C; antibodies were previously characterized (34). Tissues were stored no longer than 7 days prior to being assayed. Following 18-h incubation, sections were washed and incubated with goat anti-rabbit IgG conjugated to rhodamine. Sections were examined using immunofluorescence optics and an Olympus IX-81 microscope equipped with a digital camera for image capture.

Semi-quantitative densitometry. Average intensity per cell was utilized to assess relative levels of OGFr using Optimas software (Meyer Instruments, Inc., Houston, TX) (31). A random sample of 10 fields/section from at least 2 sections/tumor and 2 tumors/treatment were captured using a SPOT RT camera (Diagnostic Instruments, Stirling Heights, MI).

DNA synthesis rates in tumors. On the day of euthanasia, mice were inoculated intraperitoneally twice with 100 mg/kg BrdU at 6 and 3 h prior to receiving an overdose of pentobarbital. These tumors were fixed in formalin for 24 h, processed in paraffin, and sectioned. Sections were stained

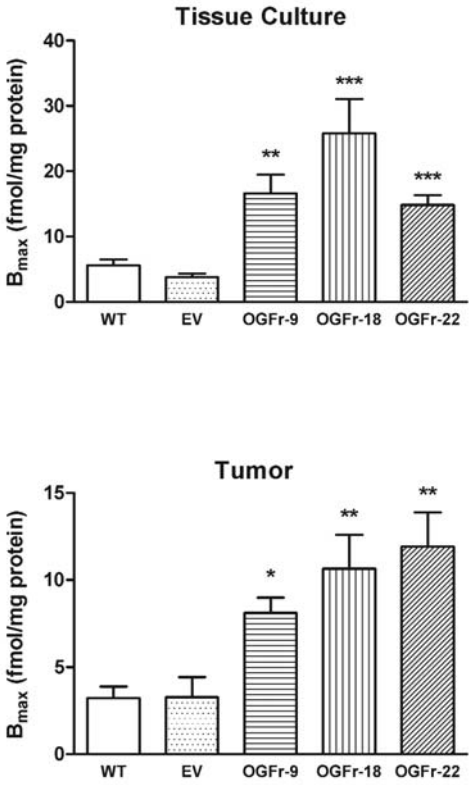

Figure 1. Transfection of OGFr in human UM-SCC-1 SCCHN cancer cells increases translational activity of the receptor in tissue culture cells and tumor. [^{3}H-Met^5]-enkephalin binding in nuclear fractions of UM-SCC-1 log-phase cells in tissue culture at 72 h after seeding, and in tumor tissues harvested at 28 days. Values (B_{max}) represent means ± SEM for 4-7 binding assays per group. Significantly different from the wild-type (WT) and empty vector (EV) group at p<0.05 (*), p<0.01 (**), and p<0.001 (***).

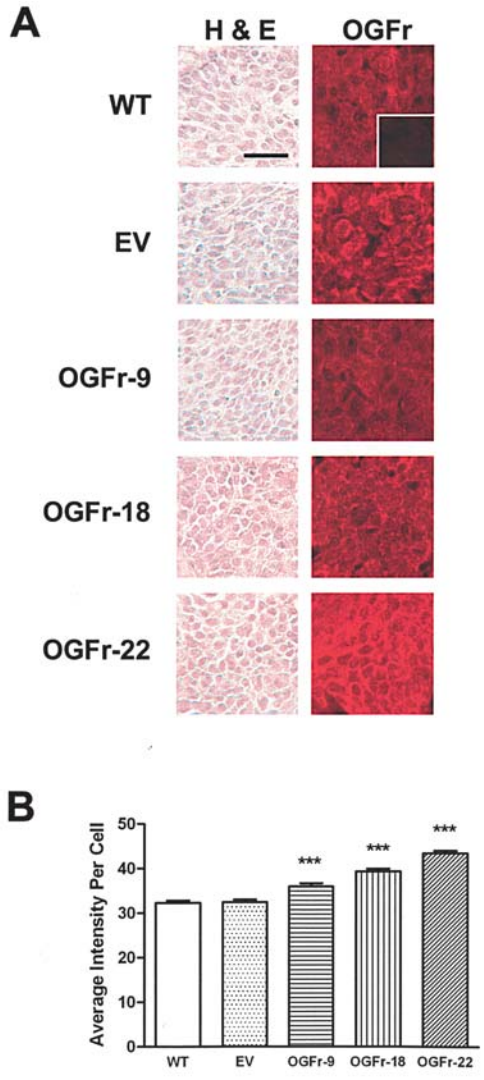

Figure 2. Photomicrographs showing overexpression of OGFr in frozen sections of UM-SCC-1 tumors. WT, EV, and OGFr-9, OGFr-18, and OGFr-22 tumors were examined on day 28 following tumor cell inoculations. (A) Sections were stained with hematoxylin and eosin (H&E) or a polyclonal antibody to OGFr. Note the increased fluorescence in specimens stably transfected with OGFr. Inset, secondary antibody only. Bar, 30 μm. (B) Quantitation of OGFr expression (average intensity per cell) in immunohistochemical preparations of tumor tissue from WT, EV, and OGFr-9, OGFr-18, and OGFr-22 specimens. Values represent mean ± SEM for at least 10 photodensitometric readings/group from 2 fields/section, 2 sections/animal/group, and 3 animals/group. Significantly different from the WT and EV groups at p<0.001 (***).

with anti-BrdU biotin conjugated (1:20) antibodies (Molecular Probes/Invitrogen, Carlsbad, CA), followed by staining with HRP secondary antibody (1:1,000) (Chemicon, Temacula, CA). The numbers of BrdU positive cells were counted in 10 random fields from 5 sections/tumor, 2 tumors/treatment group.

Statistical analyses. The incidence of tumor appearance was analyzed using χ^2 tests. Data on latency times, tumor volumes and mass, body weights, and BrdU labeling indexes were analyzed using analysis of variance (ANOVA) with subsequent comparisons using Newman-Keuls tests. In some cases, data were evaluated using two-tailed t-tests. B_{max} and K_d values for each group were analyzed using ANOVA. P-values <0.05 were considered to be significant.

Results

Overexpression of OGFr in tumor sections: immunohistochemistry and receptor binding analyses. In order to determine whether the overexpression of OGFr in SCCHN cells was maintained in the transition from *in vitro* to *in vivo*, receptor binding assays were performed to compare B_{max} and K_d values in cells and tissues (Fig. 1). The B_{max} values of clonal cells OGFr-9, OGFr-18, and OGFr-22 in tissue culture were 2.6- to 4.6-fold greater than WT (5.6±0.9 fmol/mg protein) and 3.9- to 6.8-fold greater than those of EV (3.8±0.6 fmol/mg protein) cells. Binding data for tumor tissue revealed 2.5- to 3.7-fold elevations in B_{max} levels for transfected tumors relative to both WT and EV tumors (3.2±0.6 and 3.2±1.6 fmol/mg protein, respectively). No differences between the WT or EV groups were recorded in receptor number for either cells in culture or tumor tissues. K_d values for OGFr binding studies of cells in culture (i.e., 7.2±0.7 nM) and tumor tissue (5.8±1.1 nM) were comparable among all groups of specimens.

To examine the distribution and expression of OGFr in tumor tissues, sections were stained with anti-OGFr and quantitative densitometry was employed. The location of OGFr was similar in all groups of mice (e.g., WT, EV, OGFr-9, OGFr-18, OGFr-22), with immunopositivity for OGFr being prominent in the cytoplasm, and some speckling of immunofluorescence noted in the nucleus (Fig. 2A). For all groups,

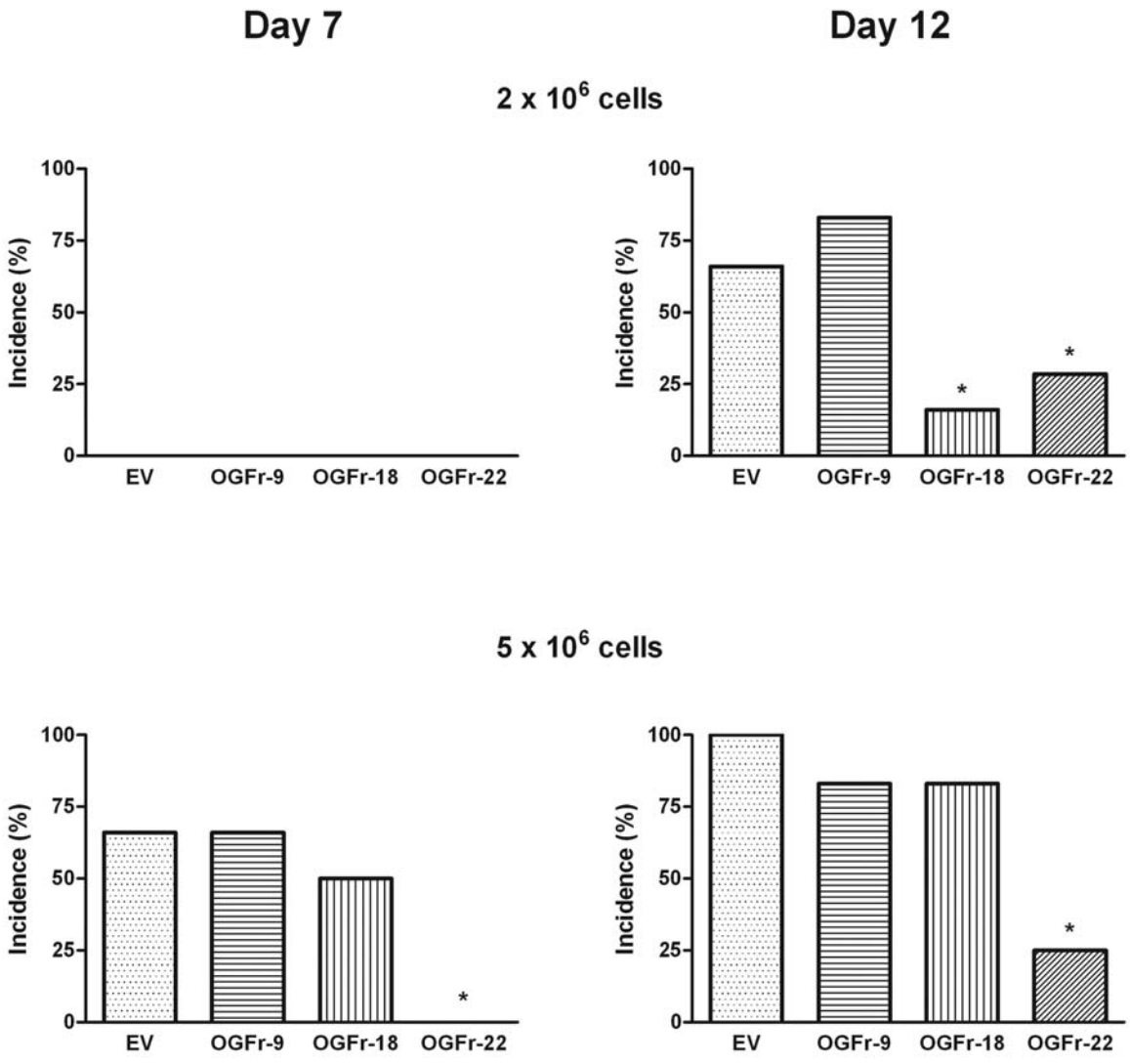

Figure 3. Transfection of OGFr in human UM-SCC-1 SCCHN cancer cells reduces the incidence of tumors and delays tumor appearance. The incidence of visible tumors (≥62.5 mm^3) at 7 and 12 days following inoculation of $2x10^6$ or $5x10^6$ cells stably transfected with empty vector (EV) or OGFr, as well as WT cells. Significantly different from the WT and EV group at $p<0.05$ (*).

tumors processed with secondary antibody showed no staining (Fig. 2A inset). Photodensitometric measurements (Fig. 2B) revealed that measurements of mean intensity per cell in specimens of OGFr-9, OGFr-18, and OGFr-22 tumors were increased 12-35% relative to that of WT and EV groups; exposure times of the WT and EV groups were similar.

Overexpression of OGFr in human SCC-1 retards tumor appearance. The incidence of measurable tumors (i.e., ≥62 mm^3) was evaluated at days 7 and 12 (Fig. 3). On day 7, no mouse receiving 2 million transfected cells had a tumor, whereas 66% of mice in the EV, OGFr-9 and OGFr-18 groups receiving 5 million cells had tumors in comparison to 0% mice receiving OGFr-22 cells.

On day 12, the incidence for a measurable tumor in animals injected with 2 million cells stably transfected with EV, OGFr-9 or OGFr-18 was 100, 70, and 83%, respectively. However, only 16 and 28% of the mice injected with 2 million OGFr-18 or OGFr-22 cells, respectively had a measurable tumor. All groups receiving 5 million cells had a tumor incidence ranging from 83 to 100%, with the exception of mice injected with OGFr-22 cells that only had a 25% tumor incidence.

Evaluation of the latency to a measurable tumor revealed that mice receiving 2 or 5 million EV cells had latencies that were 10±0.8 and 8.1±1.0 days, respectively (Fig. 4). Mice receiving 2 million OGFr-18 or OGFr-22 cells had latencies that were 3 and 10 days longer, respectively, than those for the EV group. Mice injected with OGFr-9 cells were similar in latency for tumor appearance to animals in the EV group. Mean latency for mice receiving 5 million OGFr-9 or OGFr-18 cells exhibited a latency for tumor expression that was comparable to the EV group. However, mice receiving 5 million OGFr-22 cells had measurable tumors ~10 days later than the EV group.

Overexpression of OGFr in human SCCHN cells reduces tumor weight. Tumor weights, measured on day 28, revealed significant reductions ranging from 48 to 67% for the OGFr-9, OGFr-18, and OGFr-22 groups in comparison to mice

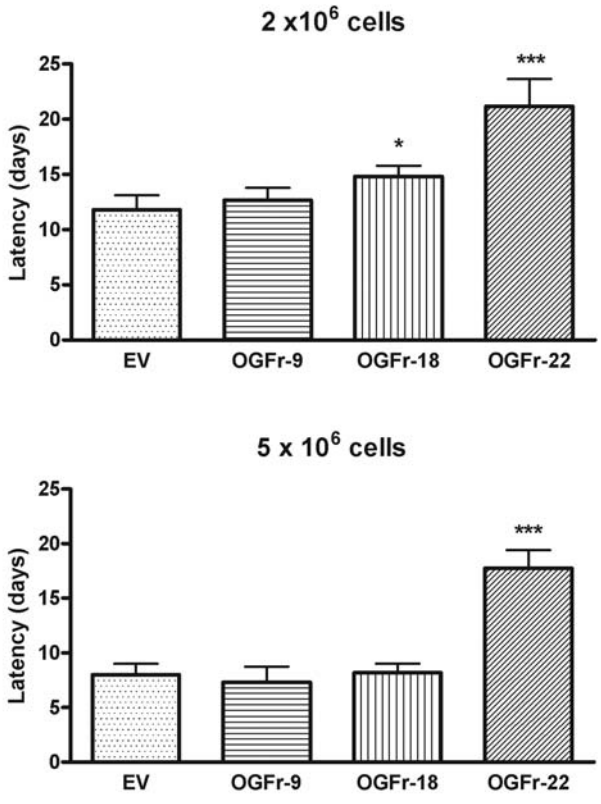

Figure 4. Latency of the appearance of visible (≥62.5 mm^3) tumors in mice receiving inoculation of $2x10^6$ or $5x10^6$ cells stably transfected with EV or OGFr, as well as WT cells. Data represent means ± SEM. Significantly different from the WT and EV group at $p<0.05$ (*) or $p<0.001$ (***).

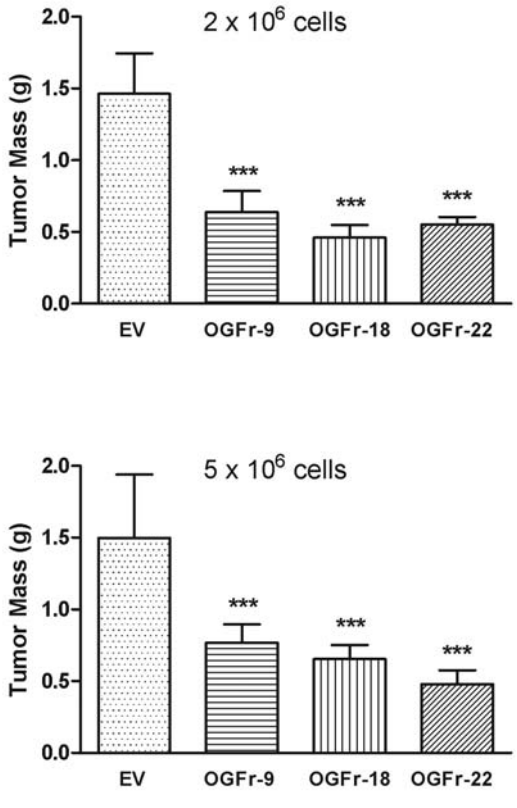

Figure 5. Tumor weight (g) 28 days after mice received $2x10^6$ or $5x10^6$ EV, OGFr-9, OGFr-18, or OGFr-22 cells. Data represent means ± SEM. Significantly different from the EV group at $p<0.001$ (***).

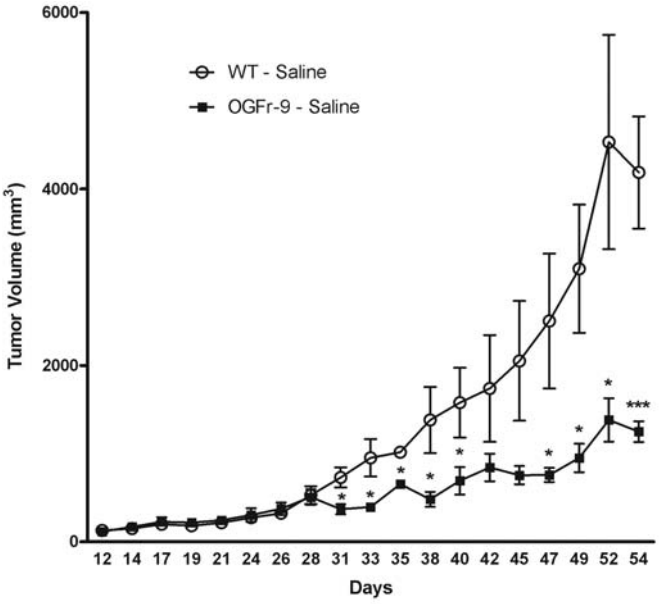

Figure 6. Measurements of tumor volume (mm^3) from days 12 to 54 after tumor inoculation of $2x10^6$ WT or OGFr-9 cells. Data represent means ± SEM for 12 mice/group. Significantly different from the WT group at $p<0.05$ (*) or $p<0.001$ (***).

receiving empty vector and injected with 2 million (1.3±0.3 g) and 5 million (1.5±0.4 g) cells (Fig. 5).

Body weights of mice receiving SCCHN cells overexpressing OGFr. On day 28, the body weights of EV mice injected with 2 and 5 million cells were 30.2±0.5 g and 33.2±1.0 g, respectively. The body weights of animals in the OGFr-9, OGFr-18, and OGFr-22 groups injected with 2 or 5 million cells were comparable to the EV animals. Moreover, no metastases were observed in organs or the body cavity at the time of death in any treatment group.

Tumor growth in animals with overexpression of OGFr. In order to examine in detail changes in the volume of tumors as a consequence of the overexpression of OGFr, and to extend the period of observation, an additional experiment was conducted with mice injected with 2 million WT and 2 million OGFr-9 cells. Latency prior to tumor appearance for the WT (12.7±1.1 days) and OGFr-9 (12.5±1.5 days) cells was comparable. However, over the course of 54 days, beginning on day 31 and continuing until day 54, tumor volumes were reduced in the OGFr-9 group by 36-70% from those neoplasias in the WT group (Fig. 6). With the exception of days 42 and 45, in which the OGFr-9 group had tumor volumes that were decreased 51 and 63%, respectively from the WT group, the reductions in tumor volume were significantly different. Examination of tumor weight on day 54 revealed that the OGFr-9 tumors (0.8±0.2 g) weighed significantly ($p<0.01$) less than tumors of the WT group (2.1±0.3 g).

DNA synthesis in human SCCHN tumors overexpressing OGFr. Assessment of the BrdU labeling index in tumors of the EV group on day 28 was 14.7±0.5% (Fig. 7). In comparison to the labeling index of the EV group, tumors of animals in the OGFr-9, OGFr-18, and OGFr-22 groups were reduced by 46-56%.

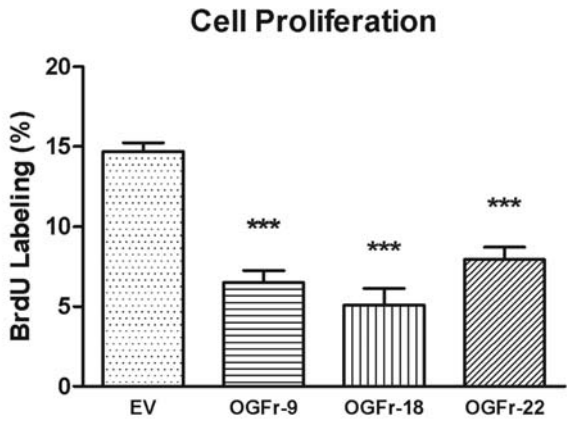

Figure 7. DNA synthesis in tumors from EV, OGFr-9, OGFr-18, and OGFr-22 cells inoculated into nude mice. Animals received injections of BrdU 3 h prior to sacrifice at 28 days. Data represent means ± SEM. Significantly different from the EV group at $p<0.001$ (***).

Discussion

The present investigation provides major insights into the molecular mechanisms of the OGF-OGFr axis in SCCHN. This is the first report that stable transfection of OGFr in human SCCHN cancer cells has a marked influence in determining the incidence and progression of disease under *in vivo* conditions. To examine whether tumor transplantation changed the properties of cells overexpressing OGFr in tissue culture, receptor binding assays were used to compare levels of OGFr in tissue culture cells and xenografts. The results showed that upregulation of OGFr was evidenced in tumors (2.5- to 3.7-fold), although some of these increases in receptor number did not reach those in cultured cells (2.9- to 6.8-fold). Moreover assessment of OGFr protein expression by quantitative densitometric measurements revealed that tumors from different clonal lines had up to 34% greater intensity corresponding to more OGFr protein than in WT or EV tumors stained with antibody to OGFr. Thus, SCCHN cells maintained an overexpression of OGFr when transplanted from tissue culture to nude mice. Moreover, cells and tumors in the EV and WT groups were similar in receptor binding assays and in quantitative densitometric measurements of OGFr staining, supporting that the vector itself was not a confounding influence and justifying the use of the EV group as the control in subsequent experiments.

Overexpression of OGFr in human SCCHN cells markedly changed the characteristics of tumorigenicity. Amplification of OGFr in tumor cells delayed the appearance of neoplasia in two of the three cell lines overexpressing OGFr dependent on dosage of cell inoculations. However, the tumors from all SCCHN cell lines overexpressing OGFr had tumor weights that were reduced ~50% or more from the EV group. In the one cell line, OGFr-9, that was followed for >50 days, tumor volume was reduced by one- to two-thirds from the EV group. Given that the OGF-OGFr axis is targeted to cell proliferation (10,12,21,22), the effect of overexpression of OGFr in SCCHN cancer cells on DNA synthesis warranted investigation as a mechanism for this action. The data show that there was ~50% decrease in DNA synthesis observed in clonal cell clines with amplified OGFr as measured by BrdU incorporation, suggesting that endogenous opioids were present in these nude mice and capable of inhibiting cell proliferation through the OGF-OGFr pathway. Thus, targeted overexpression of the OGF receptor has marked repercussions on the growth and DNA synthesis of human SCCHN cancer cells *in vivo*.

Previous studies have reported that overexpression of OGFr in human SCCHN cells increases OGFr binding and protein expression, extends the doubling time of these cells, decreases cell number and does so in a receptor-mediated and tonically active fashion; the mechanism of action involves reduction in DNA synthesis (21). The present data are consistent with these earlier observations *in vitro*, and now reveal that when transplanted into nude mice, cells over-expressing OGFr can markedly affect tumor incidence and tumor growth. Thus, the number of OGF receptors, working by way of the OGF-OGFr axis, is a determinant of the course of a deadly cancer.

The present observations on the effects of additional OGF receptors in SCCHN cells with respect to growth complement and extend those in previous studies regarding changing OGF-OGFr interactions. Corneas of rats transiently transfected with OGFr cDNA with a gene gun *in vivo* display subnormal DNA synthesis of corneal epithelial cells (24,25) and a depression in wound healing (24). We now make the important observation that stable transfections of OGFr cDNA into cancer cells that are transplanted into nude mice have marked effects on tumor response. Thus, the ramifications of transfection with OGFr cDNA have far greater implications than just altering individual cell processes.

Clinically, the OGF-OGFr axis has been recorded in biopsy and surgical specimens of human SCCHN cancer using receptor binding (9,10,15,19,20,21). Studies on the transplantation of human SCCHN cells into nude mice have revealed that the OGF-OGFr axis is functional *in vivo* (16,17,19,20). Additionally, OGFr is diminished in SCCHN tumors from patients (15), and is progressively reduced with increasing tumor burden in human SCCHN tumors transplanted into nude mice (19). Given that SCCHN is a leading cause of mortality in the world (3), and that the survival rate for this neoplasia has not improved substantially in decades (1), strategies for treatment of this lethal neoplasia are needed. In the present study, we found that introduction of additional OGFr into SCCHN cells has a dramatic effect on the expression of these neoplasias *in vivo*. An increased activation of the OGF-OGFr pathway, as indicated herein by marked reductions in DNA synthesis in SCCHN tumor cells, appears to translate into an attenuation of tumorigenic processes. Therefore, means of upregulating OGFr such as gene therapy (24,25) or agents such as imiquimod (22), perhaps with the combination of additional OGF (12,13) or chemotherapy (12), could provide a useful treatment for inhibiting tumor progression.

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