

# Progression of squamous cell carcinoma of the head and neck is associated with down-regulation of the opioid growth factor receptor

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**Abstract.** Opioid growth factor (OGF) is a native opioid peptide ([Met<sup>5</sup>]-enkephalin) that interacts with the OGF receptor (OGFr). OGF serves as a tonically active negative growth factor in neoplasia, and the OGF-OGFr axis contributes to the maintenance of an equilibrium in cell proliferation by targeting the cyclin-dependent inhibitory kinase pathway. To inquire whether the expression of OGFr is related to tumor progression, cell lines of human squamous cell carcinoma of the head and neck (SCCHN) were transplanted into nude mice, and small, medium, and large tumors were assessed for OGFr by receptor binding assays and quantitative immunohistochemistry, and for gene expression of OGFr mRNA. Large tumors had a reduction of 3- to 7-fold in OGFr binding sites relative to small tumors, and medium size tumors showed a progressive diminishment in OGF receptors that ranged between that of small and large neoplasias. Tumors with xenografts of three different cell lines of SCCHN, representing poorly- and well-differentiated cancers, exhibited similar results. Quantitative densitometric immunohistochemistry revealed data comparable to receptor binding assays. Receptor affinity and the gene expression of OGFr mRNA were unchanged in tumors of different sizes. These data demonstrate that OGFr is reduced in SCCHN with tumor progression and that translation/ posttranslation of OGFr protein, but not transcriptional levels of the OGFr gene, is (are) involved. The attenuated levels of OGFr binding capacity may serve as a marker of SCCHN. These subnormal levels of OGFr may diminish the efficacy of the OGF-OGFr axis in maintaining cell proliferative activity, and contribute to more active cell replication. Gene therapy to

reinstate more OGFr, and thus enhance OGFr function, could serve as a useful treatment for inhibiting tumor progression.

## Introduction

The opioid growth factor (OGF) ([Met<sup>5</sup>]-enkephalin) and its receptor, OGFr, have been well documented in the regulation of growth of a variety of human cancer cells in culture (1-5) and tumors in nude mice (6-9). OGF serves as an autocrine-produced, endogenous pentapeptide that inhibits cell proliferation in a receptor-mediated manner (1-5,10). OGF activity is not related to apoptosis/necrosis (11) or differentiation (12), and acts in an anchorage-independent manner (13). The mechanism of action appears to be that OGF upregulates the cyclin-dependent kinase inhibitor pathway in order to delay the G<sub>0</sub>/G<sub>1</sub> 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).

With respect to human squamous cell carcinoma of the head and neck (SCCHN), the OGF-OGFr axis has been shown to be present (4,7,16-19) and functional (4,7,16,20,21). Clinical studies assessing the level of OGFr in surgical specimens of SCCHN (19) indicated that OGFr binding sites and protein levels were 9-fold and 5-fold, respectively, less in tissues of patients with SCCHN than in normal epithelium from patients undergoing uvulopalatoplasty or tonsillectomy. Tumor margins considered to be negative for pathology revealed subnormal levels of OGFr that were intermediate between SCCHN and normal epithelium. Values of OGFr mRNA, however, were similar in SCCHN, SCCHN tumor margins, and normal specimens. These data suggest that OGFr is defective in SCCHN and that translation/posttranslation of OGF protein, but not transcriptional levels of the OGFr gene, is/are involved.

The present study examined the hypothesis that progression of SCCHN is accompanied by a decrease in OGFr protein. In order to investigate tumors of a particular size with respect to OGFr binding characteristics, an animal model that employed human SCCHN cell lines transplanted into nude mice was studied. Utilizing xenografts of 3 different SCCHN cell lines representing poorly and well-differentiated cancers, tumors were collected as 'small', 'medium', and 'large' in size (i.e., diameter, weight, volume). Receptor binding assays were performed to determine binding affinity (K<sub>d</sub>) and binding

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capacity ( $B_{\max}$ ). Immunohistochemistry, along with quantitative densitometry, ascertained the location and relative amount of OGF<sub>r</sub> in human SCCHN. Gene expression of OGF<sub>r</sub> was determined by Northern blot analysis, and quantitation was performed.

## Materials and methods

**Cancer cell lines.** SCC-1, CAL-27, and SCC-15 human SCCHN cell lines were used in this study. SCC-1 was obtained from The University of Michigan, Cancer Research Laboratory (Dr Thomas E. Carey, Director), whereas CAL-27 and SCC-15 were purchased from the American Type Culture Collection (Manassas, VA). The SCC-1 (UM-SCC-1) cell line was derived from a well-differentiated recurrent squamous cell carcinoma in the floor of the mouth of a 73-year-old male that had received irradiation (22). CAL-27 was derived from a poorly differentiated squamous cell carcinoma from the tongue of a 56-year-old male; the patient received no treatment prior to derivation of the cell line (23). The SCC-15 cell line was derived from a well-differentiated squamous cell carcinoma of the tongue of a 55-year-old male; the patient received no treatment (24). SCC-1 and CAL-27 cells were maintained in Dulbecco's MEM (modified) media, while SCC-15 were grown in 1:1 Ham's F12 and DMEM. All media were supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 U/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin). Cell cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°C. The cells were harvested by trypsinization 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 acclimatize prior to beginning experimentation.

Nude mice received a single subcutaneous injection into the right scapular region of the following:  $2 \times 10^6$  SCC-1 cells,  $2 \times 10^6$  CAL-27 cells, or  $10^7$  SCC-15 cells. For each cell line, at least 25 mice were utilized. Cell number was titrated to that which produced 100% tumors within approximately 2 weeks of cell inoculation (4,9,21,22; ATCC Product Sheet for SCC-15).

Mice were observed daily for the initial appearance of tumors. Tumors were measured twice weekly using calipers. Tumor volume was calculated using the formula,  $w^2 \times l \times \pi/6$ , where the length is the longest dimension, and width is the dimension perpendicular to length (25). Tumors were harvested as small, medium, or large when tumor dimensions were 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. Tumors were measured, removed and weighed. Tumor tissue was either flash frozen in liquid nitrogen for receptor binding assays, frozen in isopentane chilled on dry ice for immunohisto-

chemistry, 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.** For each assay, dependent on tumor size, 1-3 tumors were sampled. For SCC-1 tumors, 15 binding assays were conducted on tumors of each size whereas, for CAL-27 and SCC-15 tumors, 4 binding assays were performed on tumors of each size. Tumor tissues were removed, washed free of blood and connective tissue, immediately frozen in liquid nitrogen, and assessed following the procedures published previously (8,9,17,21). 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  $\mu$ l of [<sup>3</sup>H]-[Met<sup>5</sup>]-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 [Met<sup>5</sup>]-enkephalin. Duplicate tubes of homogenates were assayed for each concentration. Protein concentrations were determined using the Bio-Rad method with  $\gamma$  globulin as a standard.

**Immunofluorescence microscopy.** To examine for the distribution and quantity of OGF<sub>r</sub>, tumor sections were fixed and permeabilized in 95% ethanol and acetone at -20°C, rinsed in Sorenson's phosphate-buffer (SPB), and blocked with SPB containing 3% normal goat serum and 0.1% Triton X-100 at room temperature for 15 min (8,18). A chicken polyclonal antibody to OGF<sub>r</sub> (C17), generated to a recombinant OGF<sub>r</sub> protein (Cocalico Biologicals, Reamstown, PA) and fully characterized by Zagon *et al* (26), was used. Antibodies were diluted (1:500) in SPB with 1% normal goat serum in 0.1% Triton X-100, and added to sections for 18 h at 4°C. Coverglasses were washed and incubated with goat anti-chicken IgG 488 (1:1000) (Invitrogen-Molecular Probes, Carlsbad, CA) and viewed with fluorescence microscopy. Tissues incubated with secondary antibody only served as controls.

Sections were visualized using an Olympus IX-81 inverted microscope, equipped with a Diagnostic Instruments Spot RT-KE camera. Densitometric readings reflecting time of exposure were obtained from at least 15 different fields/tumor size, and were representative of at least 2 preparations. Digital images were collected from small and large tumors, with exposure times remaining constant for that of small tumors.

**Northern blot analysis of OGF<sub>r</sub> gene expression.** OGF<sub>r</sub> gene expression was assessed from at least 3 independent Northern blots, with each lane representing RNA from an individual SCC-1 tumor. 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<sub>r</sub> gene expression was performed as described earlier (19). To control for differences in the amount of RNA loaded,

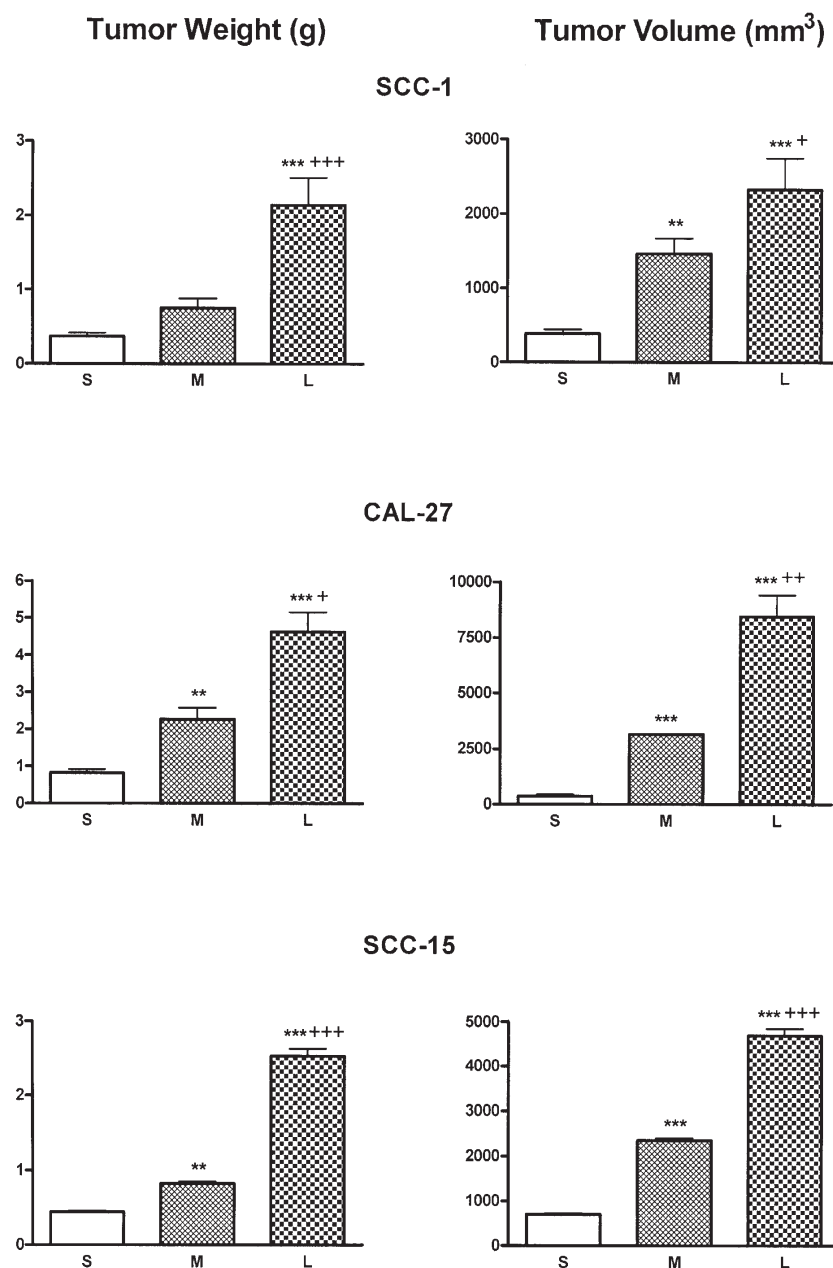


Figure 1. Tumor weight and tumor volume of small, medium, and large tumors from xenografts of SCC-1, CAL-27, or SCC-15 human SCCHN. Data represent means  $\pm$  SEM for at least 10 tumors per size. Significant difference from small tumors (\*\* $p < 0.01$  and \*\*\* $p < 0.001$ ) and from medium tumors (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).

as well as the integrity of the RNA, blots were stripped and reprobed with [<sup>32</sup>P]-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 $\alpha$  in individual blots were normalized to the OD values for GAPDH.

**Data analysis.** Data from binding assays were subjected to analysis with GraphPad Prism, and binding affinity ( $K_d$ ) and capacity ( $B_{max}$ ) values were generated by the computer program. Tumor weights and volumes,  $B_{max}$  and  $K_d$  values, and densitometric readings for gene expression, were analyzed by analysis

of variance (ANOVA); subsequent planned comparisons were made with Newman-Keuls tests. Exposure times (immunofluorescent microscopy) for small and large tumors were analyzed using a 2-tailed t-test.

## 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 injected with SCC-1, CAL-27, or SCC-15 cells. Thus, at the time of termination, mice with SCC-1, CAL-27, and SCC-15 tumors had mean body weights of  $26.4 \pm 0.9$ ,  $25.5 \pm 0.8$ , and  $27.7 \pm 0.3$  g, respectively.

Table I. Binding affinity of [<sup>3</sup>H]-[Met<sup>5</sup>]-enkephalin in nuclear homogenates of human SCCHN cells xenografted into nude mice.

| Cell line | Tumor size |         |         |
|-----------|------------|---------|---------|
|           | Small      | Medium  | Large   |
| SCC-1     | 3.9±1.5    | 1.7±0.3 | 1.7±0.3 |
| CAL-27    | 3.4±1.1    | 2.0±0.6 | 1.4±0.2 |
| SCC-15    | 4.4±1.8    | 3.2±1.0 | 3.1±1.0 |

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

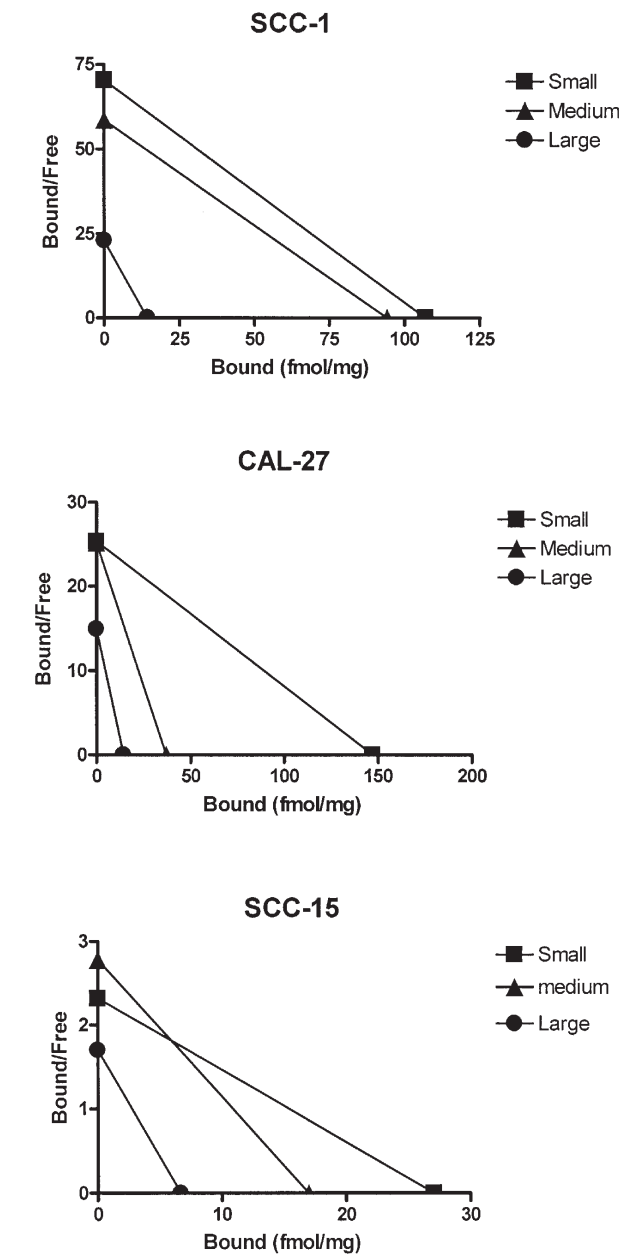


Figure 2. Representative Scatchard plots of receptor binding analyses from small, medium, and large tumors from xenografts of SCC-1, CAL-27, and SCC-15 SCCHN. Scatchard plots were generated from a single assay that approximated the mean data for the group using GraphPad Prism.

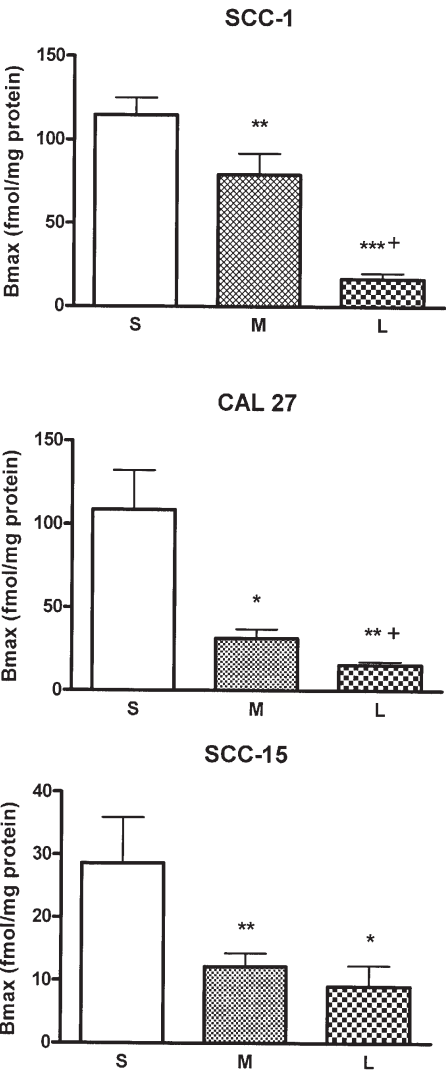


Figure 3. Binding capacity ( $B_{max}$ ) values for small, medium, and large tumors from SCC-1, CAL-27, and SCC-15 cells transplanted into nude mice. Histograms represent means ± SEM for 4-15 saturation binding isotherms. Significant difference from small tumors (\* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001) and from medium-sized tumors (\* $p$ <0.05).

**Tumor volume and tumor weight.** Tumor weight and tumor volume of small, medium, and large tumors in mice receiving SCC-1, CAL-27, or SCC-15 cells differed significantly (Fig. 1). For example, in the SCC-1 group, the weights of medium and large size tumors were 2.0- and 5.8-fold, respectively, greater than that of the small size tumors, and the large size tumors were 2.8-fold greater than those in the medium size group. Likewise, in the SCC-1 group, the tumor volume of medium and large size tumors was 3.8- and 6.0-fold, respectively, greater than that of small size tumors, and the large size tumors were 59% greater than those in the medium size group.

*OGF<sub>r</sub> receptor binding analyses*

**Binding affinity.** Binding affinity as measured by  $K_d$  values did not differ between assays for small, medium, or large tumors in any of the SCCHN cell lines (Table I and Fig. 2).

**Binding capacity.** Radiolabeled OGF binding assays revealed specific and saturable binding of the ligand to OGF<sub>r</sub> with



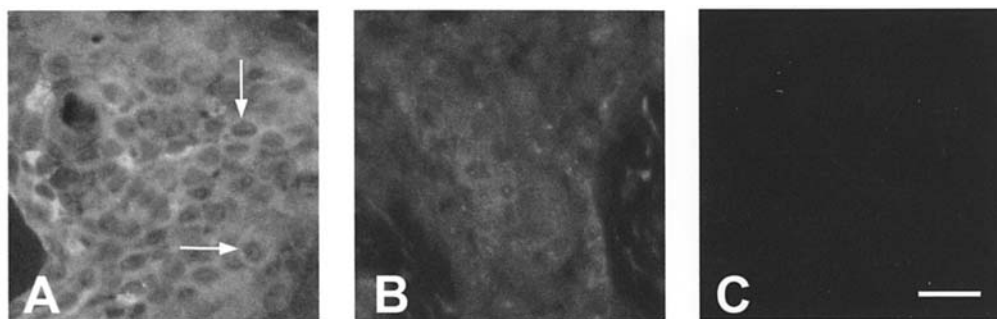


Figure 4. Immunocytochemical staining of OGFr in a small (A) and large (B) SCC-1; sections stained with secondary antibody only served as controls (C). Exposure times for all digital images were constant, and calibration was based on that for small tumors. Note intense staining of the perinuclear cytoplasm (arrows). Bar, 15  $\mu$ m.

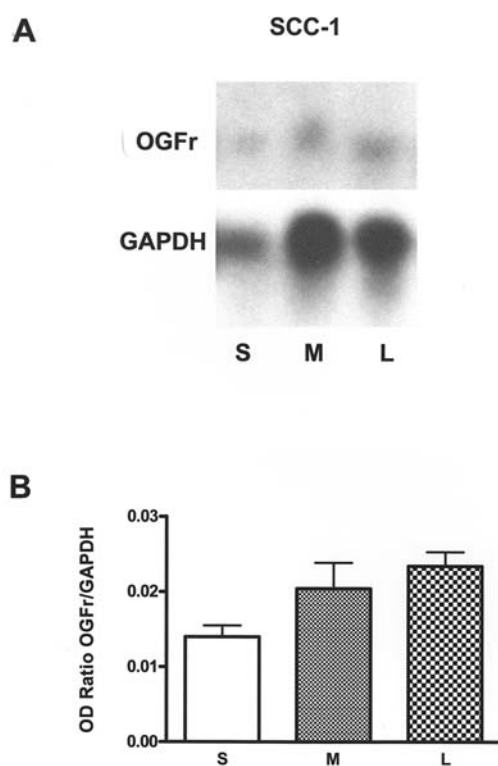


Figure 5. OGFr gene expression in specimens of small, medium, and large tumors from SCC-1 cells transplanted into nude mice. Northern blot analysis (A) of total RNA isolated from tumors probed with human OGFr cDNA; filters were stripped of isotope and reprobed with GAPDH, a constitutively expressed mRNA. Histogram of optical density ratios are presented in B. Values represent means  $\pm$  SEM. There were no statistical differences between tumors of various sizes in gene expression.

binding curves that suggested a single binding site for each of the 3 tumor cell lines examined. Values for binding capacity ( $B_{max}$ ) were dependent on the size of the tumor (Figs. 2 and 3). Medium and large tumors of SCC-1, CAL-27, and SCC-15 were reduced in the  $B_{max}$  of OGFr by 31-86% compared to small tumors, and large tumors were reduced in the  $B_{max}$  of OGFr by 26-79% compared to medium size tumors.

**Immunohistochemistry of OGFr.** OGFr immunolabeling was detected in the cytoplasm, and sometimes in the nucleus, of cells in small, medium, and large SCC-1 tumors (Fig. 4); no

immunoreactivity was observed in sections processed with secondary antibody only. Qualitative assessment indicated that immunoreactivity appeared to be greater in cells from small tumors than in cells from large tumors. Quantitative densitometry of these sections revealed that the exposure time for small tumors ( $0.86 \pm 0.04$  sec) was significantly ( $p < 0.001$ ) shorter than for large tumors ( $1.48 \pm 0.05$  sec).

**OGFr gene expression.** Quantitative analysis of OGFr gene expression as measured by RNA isolated from small, medium, or large SCC-1 tumors demonstrated similar expression profiles of OGFr normalized to GAPDH independent of tumor size (Fig. 5).

## Discussion

Data generated in this study demonstrate that the number of OGF receptors in SCC-1 corresponds to the size and, presumably, the progression of the tumor. Comparison of large tumors that were generally 2-fold greater in diameter, weight, and volume than small tumors, revealed that large tumors had 3- to 7-fold fewer OGF receptors than small tumors. However, there were no changes in the binding affinity of these receptors, indicating that a targeted change related to OGF receptor number occurred. The decrease in OGFr number was also found to be unrelated to transcriptional activity, with no irregularities in gene expression of OGFr despite the substantial difference in receptor number between small and large tumors. The question can be raised whether the down-regulation in OGFr was specific to a particular human SCC-1 cell line or if this is a more generalized observation with SCC-1. Utilization of 3 different human SCC-1 cell lines demonstrated a decrease in OGFr in all 3 cell lines as tumor size increased, indicating that the inverse relationship between tumor progression and OGFr expression appears to be a consistent property of SCC-1. The present study also questioned whether the difference in OGF receptors between small and large SCC-1 tumors occurred in poorly-differentiated and well-differentiated neoplasias. Given that the binding capacity of OGFr decreased as tumor size increased with both poorly-differentiated (CAL-27) and well-differentiated (SCC-1, SCC-15) tumors, it appears that changes in OGF receptor number with tumor progression is not dependent on the state of differentiation of the cancer.

Thus, for the first time, our results with an animal model of human SCCHN indicate that there is a loss of OGF receptors with progression of SCCHN.

The line of inquiry taken in the present investigation is based on observations reported in a previous study (19), and confirms and explains these earlier findings. McLaughlin and colleagues (19), using tissues from 64 patients with SCCHN and from 49 patients undergoing uvulopalatoplasty or tonsillectomy, found approximately one-tenth of the OGF $\alpha$  binding sites in tumor tissue in comparison with normal samples. These alterations in OGF $\alpha$  binding were unrelated to gender or the type, stage, or differentiation status of SCCHN. OGF $\alpha$  protein levels, ascertained by Western blotting with antibodies to this receptor, were reduced 5-fold in tumor tissues relative to normal epithelium. However, values of OGF $\alpha$  mRNA were comparable in tumorigenic and normal epithelium. An intriguing observation in this earlier report was that inspections of the margins of SCCHN considered to be negative for pathology revealed subnormal levels of OGF $\alpha$  relative to control specimens. The authors of this study discuss the possibility that the loss of OGF $\alpha$  in the margins may herald a transitional state directed towards tumorigenesis, with OGF $\alpha$  a sensitive prognosticator of changes from normal to abnormal epithelium. Although this previous study was suggestive that OGF $\alpha$  number was dependent on tumor progression, it must be kept in mind that 'staging' of tumors according to size was not possible. The present report employs an animal model of human SCCHN to discern the relationship between OGF $\alpha$  and SCCHN progression. The results of this study are consistent with that of the previous investigation, and demonstrate that progressive neoplasia associated with SCCHN is accompanied by a loss of OGF receptors. This defect in OGF $\alpha$  is not reliant on transcriptional changes or the state of differentiation of the tumor. It is now understandable why previous observations (19) found a subnormal number of OGF receptors in tumor margins. In actuality, these investigations were detecting a biological process of receptor loss with the advancement of these neoplasias. Therefore, if the results of these xenograft studies can be extended to humans, a principle element in the advancement of SCCHN is a loss of OGF $\alpha$ .

Since OGF is a tonically active inhibitory peptide that interacts with the OGF receptor to establish an equilibrium of cell proliferation, it can be postulated that the loss of OGF receptors in SCCHN would shift the balance of cell replication towards an increase because of the attenuation of the OGF-OGF $\alpha$  axis. This would invoke the concept of a primary participation of OGF $\alpha$  in the tumor process. Alternatively, the loss of OGF $\alpha$  with the progression of SCCHN could be secondary to other biological activities. For example, a loss of components in the synthesis of OGF $\alpha$  - or an increase in degradative process involved with OGF $\alpha$  - may be responsible for the progressive decrease in OGF $\alpha$  number with advancing SCCHN. Whether a diminishment of OGF $\alpha$  with tumor progression occurs in other types of cancers remains to be investigated.

The clinical significance of these findings is that a receptor for a native opioid peptide, which forms an endogenous opioid system that plays a role in maintaining homeostatic equilibrium in the cellular renewal of the epithelium, is compromised in advancing SCCHN. These results could be used in devising

diagnostic testing of SCCHN, particularly taking advantage of the changes in the progress of tumorigenesis. For example, examination of the margins of SCCHN for OGF $\alpha$ , in the absence of overt pathological differences, could provide valuable insight into the status of neoplasia. Future objectives based on these data include the need to investigate the mechanism underlying the defect in OGF $\alpha$  in SCCHN and develop treatment strategies that prevent the loss of OGF $\alpha$  and/or the replacement of OGF $\alpha$  (e.g., gene therapy).

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