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ORIGINAL ARTICLE

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Opioid growth factor enhances tumor growth inhibition and increases the survival of paclitaxel-treated mice with squamous cell carcinoma of the head and neck

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Abstract Paclitaxel is used as a single agent, and in combination with other drugs, as a standard of care in the treatment of squamous cell carcinoma of the head and neck (SCCHN). However, the use of paclitaxel for therapy of SCCHN may be accompanied by serious side effects. Paclitaxel is a known cytotoxic inhibitor of cell proliferation that acts by stabilizing microtubules and inducing apoptosis. Opioid growth factor (OGF), [Met^5]-enkephalin, is an endogenous peptide that has tonically active inhibitory effects on the growth of SCCHN in vitro and in vivo. OGF action is rapid, reversible, mediated by the nuclear-associated OGF receptor (OGFr), and is not cytotoxic (nor apoptotic related). The present study was designed to examine whether a combination of chemotherapy with paclitaxel and biotherapy with OGF is more effective than either agent alone in inhibiting tumor growth. Moreover, focus was placed on whether there are changes in the side effects known to occur with paclitaxel alone, following this combined therapy. Human SCC-1 cells, derived from a well differentiated SCCHN, were transplanted into athymic mice. The mice were randomized to receive intraperitoneal (i.p.) injections of sterile saline (controls), OGF (10 mg/kg, daily), paclitaxel (8 mg/kg, every other day), or both paclitaxel (8 mg/kg, every other day) and OGF (10 mg/kg, daily) beginning on the day of tumor inoculation. OGF, but not paclitaxel, delayed measurable and visible tumor appearance of mice with SCCHN. Treatment with paclitaxel, but not with other agents, had a marked effect on the body weights. Survival only was reduced in the paclitaxel group, with an average life span of 34.3 ± 3.1 days recorded, in comparison to the 50-day survival (date of termination) for all other groups. Beginning after week 4 of tumor inoculation and drug treatment, the tumor weight of the paclitaxel/OGF group was significantly reduced from the control, OGF, and paclitaxel-exposed mice. The OGFr number of the SCCHN tumors was 2.1-fold greater in the animals exposed to OGF or paclitaxel, and elevated 38% in the paclitaxel/OGF group; significant differences from the control group were found for the OGF and paclitaxel groups. These data suggest that combined chemotherapy (i.e., paclitaxel) and biotherapy (OGF) provides a valuable alternative to the standard of care for SCCHN patients.

Keywords Opioid growth factor · Opioid growth factor receptor · Paclitaxel · Chemoprotection · Nude mice

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Introduction

Cancer of the oral cavity, pharynx, and larynx accounts for 3% of new cancer cases and 2% of cancer deaths annually in the United States [6]. Worldwide, these neoplasia affect more than 500,000 individuals each year, making it the sixth most common cancer globally, and the seventh in mortality (275,000 deaths) [19]. Greater than 90% of head and neck carcinomas are squamous cell carcinomas (SCCHN) [2]. 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% [20]. The survival rate for SCCHN of the oral cavity has improved slightly (5%) in the past several decades, but the rate for SCCHN of the larynx has remained the same [6].

Currently, the most promising therapeutic regimen includes multimodality intervention of chemotherapy and radiation therapy, particularly with regards to organ preservation [23]. One of the most widely used chemotherapeutic agents, paclitaxel, has been used as a single agent and, most often, in combination with other drugs (e.g., carboplatin) [4, 12, 25, 29]. Paclitaxel is a novel taxane isolated from the bark of the Pacific yew, *Taxus brevifolia*, that is limited by toxicity, which includes neutropenia and mucositis [5].

[Met^5]-enkephalin, termed opioid growth factor (OGF), is an endogenous opioid peptide that has been shown to be an important regulator of the growth of SCCHN [13, 15, 18, 34]. OGF is a constitutively expressed native opioid that interacts with the OGF receptor (OGFr) to inhibit the growth of SCCHN and epithelial cells in vivo and in vitro [13, 30, 32–34]. The action of OGF is constitutive, tonic, stereospecific, reversible, non-cytotoxic non-apoptotic inducing, independent of serum, and occurs at physiologically relevant concentrations in a wide variety of SCCHN, including poorly differentiated and well differentiated human cell lines [13, 31]. The only opioid peptide, natural or synthetic, that influences the growth of SCCHN is OGF [13]. The action of this opioid in these neoplasias is targeted at DNA synthesis [13, 34] and is directed towards the G_0/G_1 interface of the cell cycle [34]. Gene expression and protein expression of OGF and OGFr, as well as binding activity of OGFr, have been identified and characterized in SCCHN [11, 13–16, 18, 35]. Exogenous administration of OGF has a profound antitumor action on xenografts of SCCHN [15, 18], which includes delaying tumor appearance and reducing tumor size. OGF activity in nude mice with transplanted SCCHN is receptor-mediated [15], tonic and constitutive [15], and independent to the route of drug administration [18]. Evidence has been published that OGFr may be defective in SCCHN, either as a primary or secondary pathway for this disease [16]. Translation/post-translation of OGFr protein, but not transcriptional levels of the OGFr gene, appear to be involved.

The therapeutic potential of the simultaneous use of OGF (biotherapy) and paclitaxel (chemotherapy) as an anticancer treatment for SCCHN has recently been supported by a tissue culture model [17]. Using the UM-SCC-1 cell line (SCC-1) derived from a well differentiated recurrent squamous cell carcinoma in the floor of the mouth, concomitant exposure to both OGF and paclitaxel reduced growth from control levels by 48–69% within 48 h [17]. The effect of a combination of OGF and paclitaxel on SCCHN growth in vitro was synergistic, being greater than either of the individual components. The action of OGF, but not paclitaxel, was mediated by a naloxone-sensitive receptor and was completely reversible. OGF and paclitaxel depressed DNA synthesis, whereas only paclitaxel induced apoptosis.

The present report addresses the question of whether a combination of OGF and paclitaxel influences the growth of human SCCHN in vivo, and does so beyond the efficacy of each compound. The effects of OGF and/or paclitaxel on tumor incidence, appearance, size and metastasis, and on the binding characteristics of the OGFr, were examined in a xenograft model of SCCHN using human SCC-1 cells.

Material and methods

Cell lines

The UM-SCC-1 cell line (SCC-1) [8] was obtained from the Cancer Research Laboratory at the University of Michigan (Dr. Thomas E. Carey, Director). The cells were grown in Dulbecco's MEM (modified) media 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). The cell cultures were maintained in a humidified atmosphere of 7% CO_2/93% 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.

Animals and tumor cell implantation

Male 4-week-old nu/nu nude mice purchased from Harlan Laboratories (Indianapolis, Indiana) were housed in pathogen-free isolators in the Department of Comparative Medicine at the Pennsylvania State University College of Medicine. All procedures were approved by the IACUC committee of the Pennsylvania State University College of Medicine and conformed to the guidelines established by the NIH. The mice were allowed 48 h to acclimatize prior to beginning experimentation.

The tumor cells were inoculated into nude mice by subcutaneous injection into the right scapular region. Subcutaneous injections were performed with at least 2×10^6 cells per mouse; the mice were not anesthetized for this procedure.

Chemotherapeutic administration

Four groups of mice ($n = 12$) were randomly assigned to receive i.p. injections of 10 mg/kg OGF daily, 8 mg/kg paclitaxel every other day, 10 mg/kg OGF daily plus 8 mg/kg paclitaxel every other day, or 0.1 ml of sterile saline daily. In the group receiving combined therapy, OGF was injected prior to paclitaxel. The dosages were selected based on published reports [1, 18]. Paclitaxel was dissolved in dimethyl sulfoxide (DMSO) and then

diluted in sterile saline; OGF was dissolved in sterile saline. Injections of drugs were initiated 1 h after tumor cell inoculation. Preliminary studies were performed to determine whether DMSO alone altered tumor response by injecting mice with 0.1 ml DMSO daily; no differences in tumor growth were found between injections of saline nor DMSO; thus, the data were combined for the analyses. The mice were weighed weekly to determine drug dosage.

Tumor growth and metastases

The mice were observed daily for the presence of tumors. The latency for a visible tumor to appear and the time until tumors were measurable (i.e., 62.5 mm^3) were recorded. Tumors were measured using calipers every day. The tumor volume was calculated using the formula $w^2 \times l \times \pi/6$, where the length is the longest dimension and the width is the dimension perpendicular to the length [24].

Termination day measurements

According to institutional policies and IACUC guidelines, the mice were terminated when the tumors became ulcerated or they grew to 2 cm in diameter. Fifty days following tumor cell inoculation and approximately 35–40 days following initial tumor appearance, all the mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and killed by cervical dislocation; the mice (with tumors) were then weighed. The tumors and spleens were removed and weighed, and the lymph nodes, liver, and spleen were examined for metastases.

Receptor binding analyses

The tumor tissues from some mice in each treatment group were removed at the time of death, washed free of blood and connective tissue, and were immediately frozen in liquid nitrogen. The tissues were assayed following the procedures published previously [15]. Saturation binding isotherms were generated using GraphPad Prism software; binding affinity (K_d) and capacity (B_{max}) values were provided by the computer software.

Plasma levels of OGF

At the time of termination, trunk blood was collected from several mice in each group. Plasma was separated and the OGF levels were measured by standard radioimmunoassay (RIA) procedures using a kit from Peninsula Laboratories (Belmont, California). The plasma samples were assayed in duplicate.

Statistical analyses

The incidence of tumors was analyzed by Chi-square tests. Latency for tumor appearance and tumor volume were analyzed using analysis of variance (ANOVA) with subsequent comparisons made using Newman–Keuls tests. The growth of tumors, termination day data (i.e., body weight, tumor weight, spleen weight), plasma levels of OGF, as well as the binding capacity and affinity of tumors, were compared by ANOVA and Newman–Keuls tests.

Survival data of the nude mice were analyzed using Kaplan–Meier plots. Tumor growth was analyzed using a non-linear mixed effects model for clustered data.

Results

SCC-1 tumor appearance and growth

On day 13, when 75% of the mice in the saline-injected control group had measurable tumors, 33% of the mice receiving OGF had a tumor; these values differed significantly at $p < 0.05$ (Table 1). Although fewer mice in the paclitaxel and paclitaxel/OGF groups (66% and 70%, respectively) had measurable tumors compared to the controls, these differences were not statistically significant. On day 17, when 100% of the control mice had measurable tumors, only 66% of the mice receiving

Table 1 Incidence and latency for tumor appearance of SCC-1 squamous cell carcinoma cells in nude mice treated with OGF and/or paclitaxel

Parameter	Control	OGF	Paclitaxel	Paclitaxel/OGF
N	12	12	12	10
Incidence of measurable tumor (day 13)	9/12	4/12[a]	8/12	7/10
Incidence of measurable tumor (day 17)	12/12	8/12	10/12	9/10
Latency to visible tumor (days)	7.2 ± 0.5	11.2 ± 1.5[b]	7.4 ± 1.4	8.6 ± 0.8
Latency to measurable tumor (days)	14.2 ± 0.6	17.0 ± 1.5	14.8 ± 1.7	15.5 ± 1.5

Values represent mean ± SEM (standard error of the mean)
[a]Significantly different from the control group by Chi-square analyses at $p < 0.05$
[b]Significantly different at $p < 0.02$ from controls using ANOVA

Table 2 Characteristics of nude mice 50 days after subcutaneous inoculation of SCC-1 squamous carcinoma cells and treatment (i.p.) with OGF and/or paclitaxel

Parameter	Controls	OGF	Paclitaxel	Paclitaxel/OGF
Body weight (g)	31.6 ± 0.7	32.0 ± 0.5	22.6 ± 0.8*** + + + ^^^	31.8 ± 1.1
Tumor weight (g)	2.4 ± 0.2	1.7 ± 0.2**	N.A.	0.9 ± 0.7*** + + +
Tumor volume (mm^3)	3,896 ± 535	2,590 ± 364*	N.A.	1,223 ± 238*** +
Spleen weight (mg)	243 ± 25	225 ± 12	243 ± 8	197 ± 19
Metastases	None	None	None	None

Data represent mean ± SEM. N.A. = data not available because only one mouse was alive on day 50; spleen and body weights for the paclitaxel group only were calculated on the day each mouse died. Significantly different from controls at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$(***). Significantly different from OGF group at $p < 0.05$ (+) and $p < 0.001$ (+ + +). Significantly different from the paclitaxel-treated mice at $p < 0.001$ (^^^).

OGF had tumors, and 83% and 90% of the animals in the paclitaxel and paclitaxel/OGF groups, respectively, had tumors; however, no significant differences were recorded (Table 2). All mice inoculated with SCC-1 cells developed tumors (Table 1), with 100% of the mice in the control group having tumors by day 17 and every animal in the other groups having a measurable tumor by day 28. The latency time for mice receiving OGF to develop visible tumors was 11 days, in comparison to the controls, who had a mean latency of 7 days; this 4-day delay was significantly different at $p < 0.02$ (Table 1). The mean latency time for visible tumors to appear was comparable between mice in the control group and in the paclitaxel and paclitaxel/OGF groups. The mean latency time until tumors became measurable ranged from 14 to 17 days, and did not differ between groups.

Changes in the tumor volume over the 50 days of the experiment were analyzed using a non-linear mixed-effects model for clustered data (Fig. 1). These analyses compensated for the marked loss of paclitaxel mice beginning on day 20. The tumor volumes of mice in all three treatment groups were significantly smaller than the controls. Moreover, the tumor volumes for mice receiving combined therapy were significantly smaller than the tumor sizes in the groups receiving either treatment alone.

The weights of the tumors on the termination day (day 50) in the OGF and the paclitaxel/OGF groups were reduced 29% and 62%, respectively, from the control levels (Table 2). Evaluation of the tumor volume on day 50 revealed that the OGF and paclitaxel/OGF groups had a reduction of 33% and 69%, respectively, from the control values (Table 2). As only one mouse in the paclitaxel group was alive at this time, analysis of the tumor weight or volume was performed. Measurements of the tumor weight and volume in the paclitaxel/OGF group on day 50 also revealed a decrease of 47% and

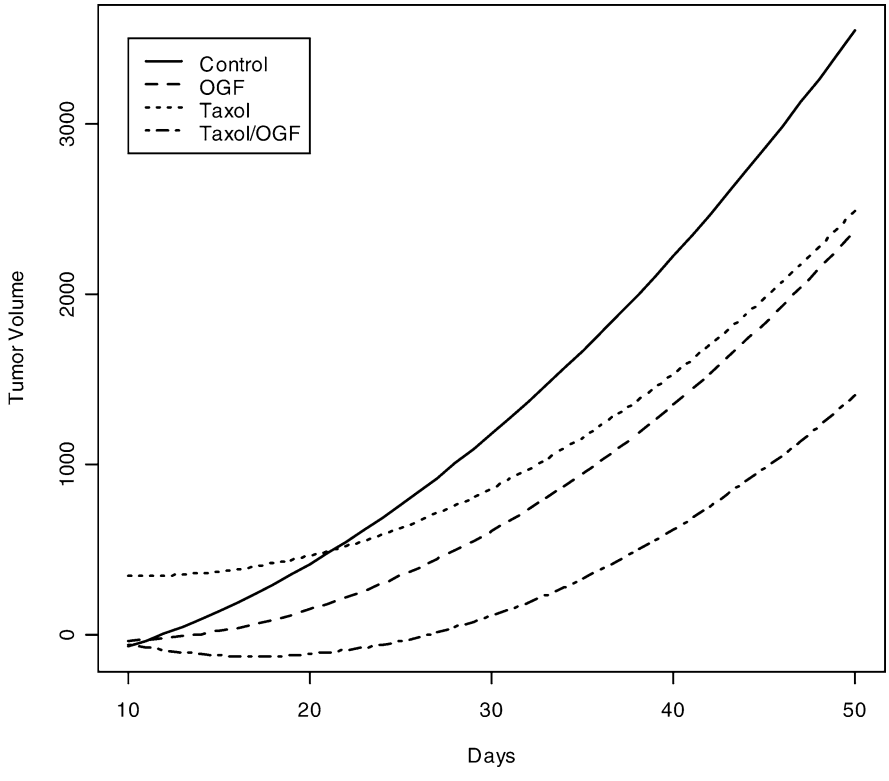

Fig. 1 Changes in tumor volume over the 50 days of the experiment, analyzed using a non-linear mixed-effects model for clustered data. These analyses were performed to accommodate the marked loss of paclitaxel mice beginning on day 20. The tumor volumes of mice in all three treatment groups were significantly smaller than the controls ($p < 0.001$). Moreover, the tumor volumes for mice receiving combined therapy were significantly smaller than the tumor sizes in groups receiving either treatment alone ($p < 0.001$). The animals were given i.p. injections of either sterile saline (0.1 ml; *Control*) daily, *OGF* (10 mg/kg) daily, paclitaxel (8 mg/kg; *Taxol*) every other day, or paclitaxel every other day plus OGF daily (*Taxol*/*OGF*)

53%, respectively, from that occurring in the OGF group.

Survival

Survival curves for mice in each group are presented in Fig. 2. Two of the 12 mice receiving paclitaxel/OGF treatment died within 1 week of initiation of the experiment; the cause(s) of these deaths appeared unrelated to tumor development or the process of injection (e.g., ulceration). Mice receiving paclitaxel began dying within 20 days of treatment. By day 40, 75% of the mice receiving paclitaxel had died, and at day 50, only one mouse in this group was still alive. One mouse in the paclitaxel/OGF group died on day 42. No mouse in the OGF or control groups died during the experimental period. Statistical comparisons of the survival curves revealed that the death rates for paclitaxel mice were statistically reliable compared to all the other groups ($p < 0.0001$). The average life span for paclitaxel mice was 34.3 ± 3.1 days in comparison to the 50-day life span of other mice (day 50 = termination day), and this difference was statistically significant from all three groups ($p < 0.001$).

Body weights and gross observations

Although all mice weighed approximately 22–23 g at the beginning of the experiment (Fig. 3), mice receiving paclitaxel had a 10% reduction in body weight at week 5 of the study and were subnormal by 9–10% on weeks 6 and 7. On the termination date (i.e., day 50), mice receiving paclitaxel weighed 28% less than the control subjects, and were significantly less in body weight than the mice in the OGF and paclitaxel/OGF groups ($p < 0.001$) (Table 2). No differences in body weights between the control animals and those in the OGF or paclitaxel/OGF groups were recorded.

Gross observations of the mice in the paclitaxel group revealed distended abdomens, impacted bowel, and severe body weight loss. Pathological reports indicated colonic dilation and peritonitis; all other organ systems appeared normal. No pathologically relevant findings could be detected for mice in the control, OGF, or paclitaxel/OGF groups.

Spleen weights did not differ among groups. In addition, no metastases were noted in the spleens, liver, or axillary lymph nodes of mice in any group.

OGFr binding characteristics

Specific and saturable binding for OGFr, with a one-site model of binding, was recorded in the tumors collected from all four groups of mice. Tumors from the paclitaxel group were obtained at days 47–50, whereas specimens from all other groups were harvested on the final day of experimentation (day 50). Binding affinity (K_d) for OGF to OGFr ranged from 1.0 nM to 2.1 nM, and did not differ among groups (Table 3). However, values for the binding capacity (B_{max}) were almost two-fold higher in

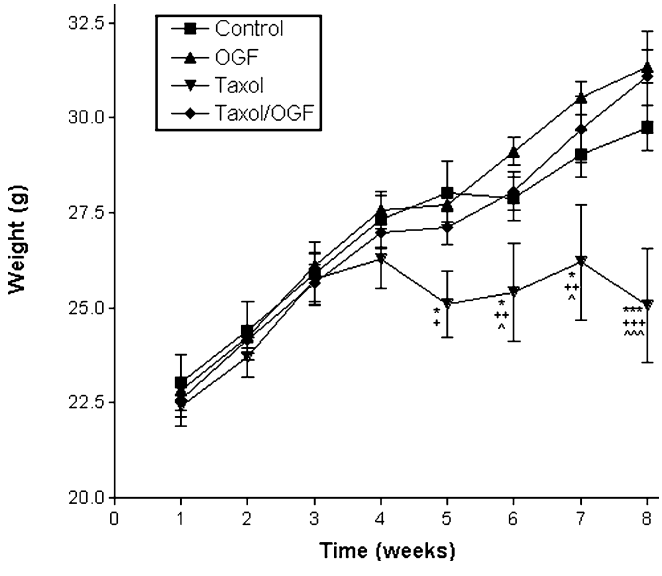

Fig. 2 Survival curves of mice inoculated with 2×10^6 SCC-1 squamous cells of the head and neck, and treated with either *OGF* (10 mg/kg, daily) and/or paclitaxel (8 mg/kg every 2 days; *Taxol*); control animals received 0.1 ml sterile saline (*Control*). Kaplan–Meier curves were analyzed and the survival of mice receiving only paclitaxel was significantly different from all other groups at $p < 0.001$

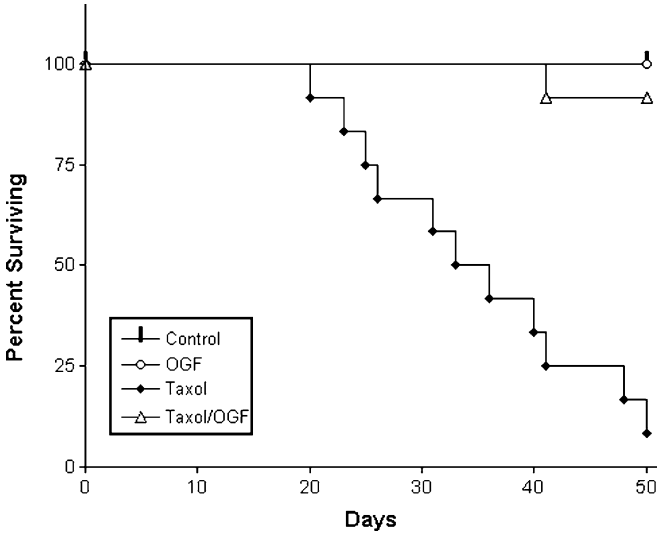

Fig. 3 Body weights of mice treated with either *OGF* (10 mg/kg, daily) and/or paclitaxel (8 mg/kg every 2 days; *Taxol*); control animals received 0.1 ml sterile saline (*Control*). The body weights were recorded every 7 days; values represent mean ± SEM. No significant differences in body weights between Control, OGF, or Taxol groups were recorded. Significantly different from the control group at $p < 0.05$ (*) and $p < 0.001$ (***), from the OGF group at $p < 0.01$ (++) and $p < 0.001$ (+++), and from the Taxol/OGF group at $p < 0.05$ (^) and $p < 0.001$ (^^^)

Table 3 Receptor binding analysis of OGFr in SCC-1 tumors from mice treated with OGF and/or paclitaxel

	Controls	OGF	Paclitaxel	Paclitaxel/OGF
K_d (nM)	1.0 ± 0.1	2.1 ± 0.3	1.2 ± 0.2	1.4 ± 0.3
B_{max} (fmol/mg protein)	14.9 ± 1.2	27.2 ± 2.2*	27.8 ± 1.6*	20.5 ± 2.1

Data represent mean ± SEM. Significantly different from controls at $p < 0.05$ (*)

the OGF plus paclitaxel group relative to the control subjects ($\backsim$15 fmol/mg protein) (Table 3).

Plasma levels of OGF

OGF levels in the plasma of nude mice bearing SCC-1 tumors ranged from 282 pg/ml to 617 pg/ml. No differences were noted between control mice with tumors and those treated with OGF, paclitaxel, or paclitaxel/OGF.

Discussion

The present results show that a combination of opioid growth factor (OGF) and paclitaxel has a potent inhibitory effect on the growth of SCC-1 in nude mice, a well differentiated human tumor model of squamous cell carcinoma of the head and neck (SCCHN). The antigrowth action of OGF and paclitaxel was synergistic, with the total inhibitory activity being greater than the sum of the parts (i.e., OGF or paclitaxel alone). This supra-additive effect of OGF and paclitaxel was most evident in measurements of tumor weight and volume. These tests performed under in vivo conditions extend earlier observations conducted in tissue culture [17], in which a combination of OGF and paclitaxel had a synergistic repressive effect on the cell number. Thus, this is the first report of the efficacy of using a combination of the biotherapeutic agent, OGF, and the chemotherapeutic agent, paclitaxel, to retard the growth of SCCHN in vivo. Although this study focused on one SCCHN cell model, SCC-1, it is known that OGF and paclitaxel influence the growth of a variety of SCCHN cell lines [10, 13]. Therefore, it is reasonable to conclude that the effects of combination therapy with OGF and paclitaxel observed herein also extend to other SCCHN cell lines.

An important observation recorded in the present investigation was the well known [7, 28] marked systemic toxicity from paclitaxel, which was manifest in significant reductions in body weight and survival, as well as gross lesions and pathological signs, and the attenuation of this toxicity by simultaneous administration of OGF. However, the amelioration of paclitaxel toxicity by OGF was not accompanied by a diminution in the antitumor action of paclitaxel. In fact, the combination of OGF and paclitaxel had an effect on tumor growth (i.e., weight, volume) that exceeded paclitaxel alone (or OGF alone). These results would suggest that chemotherapeutic levels of paclitaxel were better tolerated and were more compatible with survival when given concomitantly with the biotherapeutic agent, OGF. The alleviation of toxicity of one agent by the administration of another drug is not without precedence [3, 9]. In and by itself, the finding of protection afforded by OGF from the side effects produced by taxanes is important. However, the combination of OGF and paclitaxel could allow even higher cytostatic doses of paclitaxel to be administered in order to improve the therapeutic efficacy of this agent. Indeed, the success of chemotherapeutic agents is often limited by an intrinsic resistance of the cancer cells, and the possibility of increasing the concentration of drugs like paclitaxel without an accompanying increase in toxicity would be advantageous. Finally, it is unclear as to whether the effectiveness of a combination of OGF and paclitaxel is animal-specific and/or is due to the lack of immune components in nude mice. Because myelosuppression is a main side effect of chemotherapy, it would be valuable to explore the immunological ramifications of OGF/paclitaxel therapy in the understanding of drug mechanisms.

Previous studies have shown that surgical specimens of SCCHN have significantly fewer OGF receptors (OGFrs) than normal mucosa [16]. Translation/post-translation of OGFr protein rather than irregularities in OGFr gene transcription may be involved in this decrease in receptor number. The authors postulate that the number of OGFrs may be dependent on tumor size, and that the progressive diminishment in OGFrs in SCCHN compromises the inhibitory activity of OGF and, thereby, contributes to an accelerated cell proliferation. In the present investigation, tumor tissue from animals treated with OGF or paclitaxel and inoculated with SCCHN had over a two-fold greater binding capacity than neoplastic tissue from the control subjects. And, although not statistically significant, even those animals receiving a combination of paclitaxel and OGF had an increase of 38% in binding capacity. If the hypothesis put forth by McLaughlin et al. [18] is correct, it would be understandable that the smaller SCCHN tumors in OGF and/or paclitaxel mice would have more OGFrs (and grow slower) than those in control mice because of the repressed cell replication and less impaired OGF–OGFr axis.

Paclitaxel is a chemotherapeutic agent that prevents microtubule depolymerization, resulting in the arrest of proliferating cells in the G_2-M phase of the cell cycle that leads to cell death [31, 34]. Additionally, paclitaxel modulates a number of intracellular events that result in cellular apoptosis and ensuing nuclear degradation [27]. OGF does not influence apoptosis [31], but is targeted to the G_0/G_1 phase of the cell cycle [34]. Earlier experiments in tissue culture showed that SCCHN exposed to paclitaxel resulted in a marked increase in the number of apoptotic cells. Therefore, the mechanism for the enhanced growth inhibition in vivo by the combined effect of OGF and paclitaxel could be related to the delays in

the cell cycle (the effect of OGF), which results in the recruitment of cells into the apoptotic pathway (the effect of paclitaxel).

Paclitaxel has been reported to be active in the treatment of SCCHN, and Phase II evaluation has been successful [4]. Used as a single-agent therapy for SCCHN, this drug improved response rate, as well as median survival time, in comparison to cisplatin and 5-fluorouracil combination chemotherapy. However, 91% of the patients exposed to paclitaxel experienced neutropenia. Although OGF has been approved in Phase I trials [26], OGF has not been used clinically for the treatment of SCCHN. However, the efficacy of this compound for the treatment of SCCHN has been demonstrated in xenograft experiments [15, 18]. The present report raises the exciting potential of combining chemotherapy and biotherapy into a novel treatment modality for SCCHN. With the preclinical information that a combination of OGF and paclitaxel has a synergistic effect on SCCHN in xenografts, the prospect of clinical studies should be considered.

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