An Orthotopic Nude Mouse Model of Oral Tongue Squamous Cell Carcinoma

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ABSTRACT

Purpose: Despite advances in our understanding, prevention, and treatment of head and neck squamous cell carcinoma (SCC), the 5-year survival rates for patients remain low. This poor prognosis for head and neck SCC and SCC of the oral tongue (SCCOT) in particular reflects a limited understanding of the mechanisms of local and regional metastasis, which accounts for a majority of deaths. To analyze the molecular and cellular mechanisms of metastasis, we have developed an orthotopic nude mouse model of SCCOT.

Experimental Design: Nude mice were injected submucosally in the tongue or subcutis with human squamous cell carcinoma of the oral cavity cell lines Tu159, Tu167, and MDA1986. The mice were necropsied and examined for the presence of primary tumors, and regional and systemic metastases.

Results: For all three of the squamous cell carcinoma of the oral cavity cell lines, tumors developed more readily in the orthotopic site, the tongue, than in the ectopic subcutis. MDA1986 cells were highly tumorigenic, particularly at the orthotopic site, with as few as 5 × 10^3 cells producing tumors in all of the mice. In contrast, s.c. tumor formation required at least 1 × 10^5 cells. The tumorigenicity observed between those mice given submucosal inoculation and those mice given s.c. inoculation (P < 0.0001). Regional metastases initially occurred in <10% of mice. To generate tumor lines of increased metastatic potential, regional metastases were isolated from cervical lymph nodes after the development of orthotopic tongue tumors. Serial passage of these lymph nodes resulted in a cell line more metastatic than its parental line. When injected into the tongues of mice, these cells metastasized to regional lymph nodes in 30% of mice and to the lungs in 20%.

Conclusions: In this orthotopic murine model, oral tongue cancer recapitulates the behavior of human SCCOT, allowing for detailed studies of its biology and therapy.

Introduction

HNSCC remains a significant public health problem (1). Cancers of the oral cavity and pharynx alone account for 363,000 new cases worldwide and almost 200,000 deaths annually (2). In 2001, these tumors are predicted to account for 30,100 new cancers, representing 3% of all cancers in the United States, equal in incidence to leukemia and greater than all of the endocrine tumors. In particular, SCCOT is among the most common tumors of the head and neck (3). Despite advances in our understanding, prevention, and treatment, the 5-year survival rates for patients with SCCOT have not improved in the past 25 years and remain ~50%. This high mortality is considerably worse than rates for breast, cervical, or colorectal cancer (3). This poor prognosis for SCCOT may reflect a limited understanding of the mechanisms of local and regional metastasis, which accounts for a majority of deaths.

Regional metastasis strongly correlates with tumor recurrence and mortality (4–8). Extension of cervical metastases outside the lymph node capsule (extracapsular spread) is an even worse clinical indicator (9, 10). In a recent retrospective review of >250 patients from 1980–1995 with SCCOT, overall and disease-specific survival rates for patients with extracapsular spread were 30% and 50%, respectively (11). Distant metastasis developed in 24.4% of patients with regional metastasis but in only 3.3% of patients without neck disease.

Despite these abundant data, little is known regarding the tumor biology of regional metastases of SCCOT. Furthermore, there is no reliable animal model for study. Therefore, we have developed an orthotopic model of SCCOT metastases by injecting cell lines from human oral cavity SCC into the tongue of nude mice. In this model, local tumor growth, and regional and distant metastases demonstrated a histopathological similarity to SCCOT primary tumors from patients. Finally, these tumors recapitulate the regional and distant metastatic patterns seen in patients with SCCOT.

MATERIALS AND METHODS

Cell Lines

Three lines of human SCCOC (Tu167, Tu159, and MDA1986), established from freshly resected human tumors

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2 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; SCC, squamous cell carcinoma; SCCOT, squamous cell carcinoma of the oral tongue; GFP, green fluorescent protein; SCCOC, squamous cell carcinoma of the oral cavity.
(12) were obtained from the laboratory of Dr. Gary L. Clayman, The University of Texas M.D. Anderson Cancer Center. B16-BL6 melanoma cell lines were used to determine the feasibility of the orthotopic injection procedure and have been described previously (13–15).

**GFP Transfection of SCCOC Cell Lines and Selection for GFP Expression**

PEGFP-C1 plasmid (Clontech, Palo Alto, CA) was transfected into the cell lines by the CaPO4 method using 2 m CaCl2 and 2 × Hanks’ balanced, buffered solution (Calbiochem, San Diego, CA). The cells were plated onto 100-mm dishes at a density of 1 × 10^6 cells/dish. The cultures were incubated after transfection at 37°C in an incubator under 5% CO2 for 16 h; cultures were then washed and refed with fresh DMEM (high glucose) supplemented with 10% fetal bovine serum, L-glutamine, nonessential amino acids, penicillin-streptomycin, sodium pyruvate, and vitamins. To select clones with resistance to neomycin, 400 µg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) in DMEM was added starting 48 h after transfection. Two weeks later, the G418-resistant clones were expanded, and 2 × 10^6 cells/cell line were analyzed on a flow cytometer at the highest excitation and emission wavelengths to view GFP. The top 5% of fluorescent cells were isolated and cultivated at 37°C in a 5% CO2 incubator.

**Animal Care and Injections**

Male athymic nude C57BL/6 mice, 6–10 weeks of age, were purchased from the National Cancer Institute (Bethesda, MD) and housed in a specific pathogen-free animal facility. The animals were fed irradiated mouse chow and autoclaved reverse osmosis treated water. All of the animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Usage Committee. To determine whether these human tumor cell lines would grow better when implanted at an orthotopic site, i.e., a tissue bed most closely resembling the primary site of origin, we injected SCCOC in both the tongues and flanks of the experimental animals. All of the mice were anesthetized with sodium pentobarbital (50 mg/kg body weight). Cells from all four of the tumor lines (B16-BL6, Tu167, Tu159, and MDA1986) were then prepared in 50 µl of Hanks’ balanced, buffered solution in the following dilutions: 1 × 10^3, 5 × 10^3, 1 × 10^4, 5 × 10^4, 1 × 10^5, and 5 × 10^6 cells. A group of five mice were used for each concentration.

**Subcutaneous Flank Injection Technique**

In these groups, each mouse underwent s.c. injection of cells (suspended in a volume of 50 µl) directly into the flank using a 1-ml tuberculin syringe (Hamilton Co., Reno, NV) with a 30-gauge hypodermic needle. Mice were then examined every other day for tumor development. When present, tumors were measured using calipers in cephalad-to-caudal and left-to-right dimensions. The maximal tumor diameter was recorded.

**Orthotopic Sublingual Injection Technique**

In these groups, each mouse then underwent submucosal injection cells (suspended in a volume of 50 µl) directly into anterior tongue using a 1-ml tuberculin syringe (Hamilton Co.) with a 30-gauge hypodermic needle. Mice were then examined every other day for the development of tongue tumors and weight loss. The mice were killed by CO2 inhalation when they had lost > 25% of their preinjection body weight.

**In Vivo Selection of Cell Lines for Increased Metastatic Potential**

Using a similar technique, a GFP-expressing Tu167 SCCOC cell line, Tu167GFP was injected into the tongues of nude mice. Regional metastases developed, and the mice were killed using CO2 inhalation. A standard apron-flap incision was made in the neck of the experimental animal. Lymph nodes were harvested by aseptic techniques, dissociated mechanically using a wire mesh sieve, and placed into culture. Cells were grown in DMEM with 20% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin (Life Technologies, Inc.). To inhibit fibroblast overgrowth of cultures and allow selection of tumor cells, 300 µg/ml G418 were added to the cultures. These tumor lines were harvested within three to five in vitro passages and reinjected into the tongues of athymic nude mice. These cells were then injected submucosally into the tongue of several nude mice. The mice were killed after they had lost 25–30% of their body weight. The cervical lymph nodes were harvested by sterile dissection techniques and placed in antibiotic containing medium. The lymph nodes were dissociated, and tumor cultures were cultured for several weeks (as a monolayer) in a 5% CO2 incubator at 37°C until colonies of epithelial cells could be identified. To select for the growth of cells that had been transfected previously with GFP and the neomycin phosphotransferase gene, the cells were then grown in medium containing G418 (400 µg/ml). The neomycin-resistant cells were expanded and designated as the Tu167G-LN1 cell line. Tu167G-LN1 cells were then injected into the tongue of a group of nude mice (n = 10), which were killed when they lost > 25% of initial body weight. Cervical lymph nodes from 2 mice were sterilely resected and grown in tissue culture to generate the Tu167LN2 cell line. The cervical lymph nodes of the remaining 8 mice and the lungs of all 10 of the mice were fixed in formalin, embedded in paraffin, and evaluated by light microscopy after staining with H&E.

** Necropsy and Tissue Preparation**

After the mice were killed, the tongues, cervical lymph nodes, and viscera (lungs, liver, and brain) were removed and placed in formalin solution overnight. Each specimen was embedded in paraffin and sectioned. Sections were stained with H&E and evaluated by light microscopy for the presence of regional or distant metastases.

**Microscopy**

A Leica (model MZ FLIII) fluorescence dissecting stereomicroscope was used to visualize fluorescent metastases. The microscope was equipped with a 100-W, mercury vapor lamp power source and fitted with a GFP filter set. Images were processed using Image Pro Plus (version 4.0; Media Cybernetics, L.P., Silver Spring, MD) and Adobe PhotoShop (version 5.5; Adobe Systems Inc., San Jose, CA).
Statistical Analyses

Tumorigenicity of MDA1986 SCCOC. For this pilot study, standard maximum likelihood based methods were not applicable. Exact logistic regression was used to assess both the tumorigenicity of SCCOC in mice and the effects of orthotopic (submucosal) versus ectopic (s.c.) tumor development. Four to five mice were inoculated at each level/dilution combination (56 mice total). The regression model included terms for the dilution effect (ranging from $1 \times 10^3$ to $5 \times 10^6$ cells) and for the inoculation location (submucosal versus s.c.). The $P$s for the location effect were evaluated against an $\alpha$ significance level of 0.05.

Selection of Human SCCOC Cell Line Tu167 for Greater Metastatic Potential. Fisher’s exact test and cross-tabulation was used to compare the rates of regional and lung metastasis in 45 mice inoculated with the Tu167 cell line versus 10 mice injected with Tu167GLN1 cell line. The $p$ value for this test was evaluated against an $\alpha$ significance level of 0.05.

All of the statistical computations were performed on a Dell 600 mHz PC using the SAS statistical system and S-plus (Cary, NC).

RESULTS

Human SCCOC Cell Lines Form Tumors when Injected Submucosally into the Tongues of Nude Mice. The highly metastatic B16-BL6 melanoma cells, implanted into the tongues of the syngeneic C57BL/6 mice, formed tumors in the tongues of all of the injected mice and yielded high rates of regional metastases (Fig. 1A and B), demonstrating the feasibility of this orthotopic (submucosal lingual) injection technique. Next, we injected three different human SCCOC cell lines (MDA1986, Tu159, and Tu167) into the tongues of nude mice. The injection of $5 \times 10^5$ cells produced tongue tumors in all of the injected mice, regardless of the cell line used. The orthotopic SCCOC tumors resulting from submucosal injection of Tu167 (Fig. 1C) were very similar in histological appearance to that of primary human SCCOT specimens (Fig. 1D), demonstrating keratin pearls and intercellular bridging.

Tumorigenicity in Orthotopic and Ectopic Organs. For the three human SCCOC cell lines, a spectrum of tumorigenicity was observed related to tumor cell line, concentration, and site of injection. These trends are described below.

SCCOC Tu159. Tongue tumors developed in all of the mice injected with $5 \times 10^2$, $1 \times 10^3$, $5 \times 10^3$, $1 \times 10^4$, and $5 \times 10^4$ Tu159 cells. s.c. tumors developed in none of the animals inoculated with $5 \times 10^3$ cells and in only one of five animals and three of five animals inoculated with Tu159 at $1 \times 10^3$ and $5 \times 10^3$ cells, respectively. All of the mice injected with $1 \times 10^5$ and $5 \times 10^5$ Tu159 cells developed s.c. tumors. These data show that the Tu159 cells are more tumorigenic at the orthotopic site than the ectopic s.c. tissue.

SCCOC Tu167. In the mice injected with Tu167, tongue tumors were found in all of the animals injected submucosally with at least $1 \times 10^5$ cells. However, only one of five mice developed s.c. tumors when injected with $1 \times 10^3$, $5 \times 10^3$, or $1 \times 10^4$ cells, indicating at least a 50-fold difference in the minimal tumorigenic dose between the tongue and subcutis (Table 1).

SCCOC MDA1986. The MDA1986 cell line was highly tumorigenic, particularly at the orthotopic site, with as few as $5 \times 10^3$ cells producing tumors in all of the mice. In contrast, s.c. tumor formation required an injection of at least $1 \times 10^5$ cells. The results of the tumorigenicity study of the MDA1986 cell line are summarized in Table 1. Furthermore, in the exact logistic regression model the difference in tumorigenicity observed between those mice given submucosal inoculation and those mice given s.c. inoculation was statistically significant ($P < 0.0001$).

The Metastatic Pathways of Human SCCOT Are Recapitulated in the Orthotopic Nude Mouse Model of SCCOT. For mice injected sublingually with the Tu167 tumor cells, only 4% developed lymph node metastasis, and none of these animals had detectable pulmonary metastases. Two methods were used to identify cervical and distant metastasis. Immunohistochemical staining with an anticytokeratin antibody (data not shown) was performed to confirm the epidermoid character of each metastasis.

Selection of SCCOC Cell Lines for Greater Metastatic Potential. This initially low rate of regional metastases for Tu167 provided us with a model system for characterizing the biological processes and specific molecules critical for the development of cervical and systemic metastases. Our objective was to generate tumor lines of increased metastatic potential by identifying regional metastases within the small, cervical lymph nodes of mice after the development of orthotopic tongue tumors.

At first, it was quite difficult to identify these uncommon metastases, in part because the unique location of the tongue leads to rapid weight loss, necessitating early sacrifice. To identify microscopic regional metastases earlier, the Tu167 cell line was transfected with GFP, and the resultant cell line, Tu167 tumor cells expressing GFP, was orthotopically implanted into the tongue of the nude mouse. Visible lingual tumors developed, and the mice were sacrificed after $>25\%$ preinjection weight loss. At necropsy, fluorescence stereomicroscopy was used to identify metastatic GFP-transfected tumor cells. This method readily identified both primary tongue tumors (Fig. 1E) and submandibular metastasis of GFP-expressing Tu159 (Fig. 1F). Both of tumors were confirmed histologically (Fig. 1G).

Metastatic tumor cells from lymph nodes containing a fluorescent tumor were harvested and grown in culture. The resulting tumor cell line, Tu167G-LN1, was expanded and re-injected into the tongues of 10 mice. With this new cell line, cervical (Fig. 1H) and distant metastases (Fig. 1I) were seen with greater frequency (see Table 2). Using Fisher’s exact test, there was a statistically significant difference in regional and lung metastases rates between Tu167 and Tu167G-LN1 ($P = 0.0004$).

DISCUSSION

Treatment failure in HNSCC results primarily from regional and distant metastasis. To study the mechanism of metastasis in SCCOT, we have developed a reliable, orthotopic nude mouse model. First, we verified the feasibility of sublingual injection and the development of regional metastases using the pigmented B16-BL6 melanoma cell line in
syngeneic mice. Next, three different human SCC oral cavity tumor lines were then injected submucosally into the tongues of nude mice: Tu167, derived from a primary tumor of the floor of mouth; Tu159, from a primary SCCOT; and MDA1986, cultured from a cervical metastasis of human SCCOT. All of the tumor lines were more tumorigenic in the orthotopic site than in the subcutis. This nude mouse model of oral cavity cancer substantiates the orthotopic principle that tumor cells grow better in their tissue of origin than in an ectopic (s.c.) site.

The orthotopic nude mouse model also provides an invaluable method for in vivo selection and generation of tumor cell lines of greater regional and distant metastatic potential. Initially, only 4.4% of mice injected with Tu167 developed cervical lymph node metastases. After serial passages, the more metastatic cell line Tu167G-LN1 was identified. When orthotopically reimplanted, Tu167G-LN1 cells produced a 30% rate of regional lymphatic metastasis. Pulmonary metastasis did not develop in mice injected with Tu167 cells, whereas 20% of the mice injected with Tu167G-LN1 had metastases in the lungs.

Fig. 1  A and B, regional metastasis from orthotopic sublingual implantation of B16-BL6 melanoma. The tongues of C57BL/6 black mice were inoculated with syngeneic B16-BL6 melanoma cells, and mice were killed after 14 days and necropsy performed. An obvious tumor is seen in the tongue. Bilateral metastases are easily identified by melanotic pigmentation within the cervical lymph nodes. C and D, comparative histopathology showing the resemblance between orthotopic oral tongue cancer in the nude mouse and human SCCOT. Photomicrographs of (C) the Tu167 human SCCOC cell line grown orthotopically in the tongue of nude mouse and (D) a human SCCOT tumor. E–G, visualization of orthotopic tongue tumor and regional metastasis in situ with GFP. Tu159GFP was inoculated submucosally in the tongue of each mouse; the mouse was killed, and the tumor was visualized under a dissecting fluorescence microscope. The primary tumor in the tongue (E) is readily apparent. An area of fluorescence, noted in the submandibular region (F), was resected, revealing metastatic tumor on microscopic examination (G; H&E). H, regional metastasis of the orthotopic TU167G-LN1 SCCOC. Photomicrograph of a cervical lymph node from a nude mouse in which the Tu167G-LN1 line was grown orthotopically, showing a subcapsular metastatic tumor (H&E). I, distant pulmonary metastasis from the orthotopic TU167G-LN1 SCCOC. Photomicrograph of a lung from a nude mouse in which the Tu167G-LN1 line was grown orthotopically, revealing distant metastatic tumor (H&E).
We are continuing this in vivo passage for several more cycles to select for cell lines of greater metastatic potential. By developing SCCOC cell lines with low and high metastatic propensity, it may be possible to identify the molecular basis of metastasis in SCCOT.

An orthotopic nude mouse model to investigate the cellular and molecular mechanisms of metastasis in human neoplasia was first described by Fidler et al. (15, 16) and Killion et al. (17). Other groups using human solid tumors from a variety of organ sites have subsequently documented its significance (18, 19). The orthotopic implantation of tumor cells restores the correct tumor-host interactions, which do not occur when tumors are implanted in ectopic s.c. sites (15). Orthotopic models can properly evaluate the metastatic propensity of human tumors and select cell lines of varying metastatic potentials. In addition, an accurate experimental animal model is necessary to evaluate the efficacy of novel therapies (17, 19).

Previous orthotopic models of oral cancer have met with limited success. The first report of orthotopic growth of human oral cancer tumor lines in nude mice was published by Fitch et al. (20), who aspirated cells from fresh human tumors growing s.c. in nude mice and injected them into the tongues of nude mice. These studies showed an equal tumorigenicity in the oral cavity and the subcutis. Certain SCCOT cell lines that grew better in the ectopic subcutis of the nude mouse may have accounted for these results.

An oral cavity model, developed by Dinesman et al. (21), relied on transcutaneous injection via a submandibular route into the tissue deep to the mylohyoid muscle beneath the floor of mouth. Whereas only 5% of these tumors metastasized to regional lymph nodes, 40% of animals developed pulmonary metastases. Whereas recent reports using this model have highlighted its potential utility in quantifying local invasiveness into muscle and bone (22, 23), the injection of cells transcutaneously into the deep neck muscles differs from submucosal injection. Tumor seeding and spillage may predispose these animals to develop hematogenous pulmonary metastasis, contradicting an orthotopic approach. This may account for the higher incidence of pulmonary metastasis compared with regional lymphatic metastasis.

An orthotopic model of cutaneous SCC metastases was developed by Chen et al. (24), using a transformed skin keratinocyte line that was injected s.c. in syngeneic mice. After passage in vivo before harvesting metastatic variants from the lymph nodes, the resultant cell lines expressed higher levels of chemokine growth-regulated oncogene-α, interleukin-8, and nuclear factor kB than the parental cells (25). Our mouse model offers an ideal and analogous experimental system to study the molecular basis of metastasis in mucosal HNSCC.

Orthotopic nude mouse models provide invaluable insights into metastasis, both at the molecular and cellular level, and are a vital first step in identifying safe and effective new therapies. However, nude mouse models have limitations. Data acquired should be additionally substantiated with complementary studies with immunocompetent models and analysis of archival human, oral tumor specimens. All of these studies provide critical information necessary for preclinical assessment of new drugs to fight head and neck cancer.

In summary, we have developed a model of SCCOT to study regional lymph node metastases and distant visceral metastases. This model should facilitate in vivo studies of the systemic cellular and molecular mechanisms of tumorigenicity, growth, and metastasis in SCCOT. Whereas no animal model can correlate directly with the human metastatic process, this orthotopic animal model will play an important role in the evaluation of novel therapies for the treatment of HNSCC.

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