An Orthotopic Model of Anaplastic Thyroid Carcinoma
in Athymic Nude Mice

Seungwon Kim,1 Young-Wook Park,1 Bradley A. Schiff,1 Dao D. Doan,1 Yasemin Yazici,1 Samar A. Jasser,1 Maher Younes,1 Mahitosh Mandal,1 Benjamin N. Bekele,2 and Jeffrey N. Myers1

Departments of 1Head and Neck Surgery and 2Biostatistics, University of Texas M.D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Purpose: To develop an orthotopic model of anaplastic thyroid carcinoma (ATC) in athymic nude mice.

Experimental Design: Various thyroid carcinoma cell lines were injected into the thyroid gland of athymic nude mice to determine whether such injection was technically feasible. ATC cells were then injected into the thyroid gland or the subcutis of nude mice at various concentrations, and the mice were then followed for tumor development. The tumors were examined histopathologically for local invasion or regional or distant metastasis.

Results: Injection of tumor cells into the thyroid glands of nude mice was technically feasible and resulted in the formation of thyroid tumors. The ATC cell line DRO showed significantly higher tumorigenicity in the thyroid gland than in the subcutis. In contrast, oral squamous cell carcinoma cell line TU167 shows no significantly higher tumorigenicity in the thyroid gland than in the subcutis. ATC tumors established in the thyroid gland also produced symptomatic compression of the esophagus and the trachea. Local invasion of the larynx and trachea was as well as high rates of pulmonary metastasis were also observed. Immunohistochemical staining showed higher microvessel density as well as higher expression of vascular endothelial growth factor and interleukin-8 in the thyroid orthotopic tumors than in ectopic tumors.

Conclusion: An orthotopic model of ATC in athymic nude mice was developed that closely recapitulates the clinical findings of human ATC. This model should facilitate the understanding of the pathogenesis of ATC and aid in the development of novel therapies against ATC.

INTRODUCTION

Anaplastic thyroid carcinoma (ATC) is one of the most fatal human malignancies known, with a median overall survival of 6 months following diagnosis (1). Although it only accounts for 2% of all thyroid carcinomas, its highly aggressive clinical course and uniformly fatal outcome set this disease apart from well-differentiated thyroid carcinomas such as follicular or papillary thyroid carcinoma (2). ATC occurs most frequently in the elderly and presents as a rapidly enlarging neck mass. It is characterized by its rapid progression of local disease and a very high incidence of regional and distant metastasis. The mean tumor size at the time of diagnosis is already ~8 cm (3). Furthermore, 40% and 50% of patients already have regional and distant metastasis, respectively, at the time of diagnosis (4).

Inherent in the extremely poor prognosis of patients with ATC is the absence of any effective curative modality against this disease. Although surgical resection, chemotherapy, or radiation therapy may be employed in the treatment of ATC, they are most often used with palliative rather than curative intent. The development of effective curative treatments for patients with ATC has been hampered by its relative rarity. Another major hurdle in this endeavor has been the lack of an appropriate animal model in which potential therapeutic strategies against ATC may be evaluated. Most current animal models consist of establishing s.c. tumors in nude mice. However, it has been shown that the microenvironment of the subcutis is radically different from that of the organs where tumors originate (5–7). This difference manifests in the different biological behavior of the tumors in orthotopic versus ectopic locations (8–11). Indeed, tumors in orthotopic locations have been shown to have significant differences in drug response compared with tumor established s.c. (9, 10). Furthermore, s.c. tumors in mice fail to display metastatic patterns reflective of the original human tumors (7). In contrast, orthotopic models accurately replicate the metastatic process and have proven to be valuable for selecting populations of tumor cells with increased metastatic potential via serial passages of lymph node metastases (12, 13).

It is now well established that orthotopic tumor cell implantation in nude mice is the method of choice for modeling human cancers because this model allows the evaluation of treatment strategies in a biological setting that closely mimic the disease process in humans. To facilitate the investigation of effective therapeutic strategies for ATC and to better understand its mechanisms of tumor progression, we have developed an orthotopic model of ATC in nude, athymic mice. This model closely replicates the clinical behavior of ATC in humans, including rapid tumor growth, tracheal and esophageal compression, laryngeal and tracheal invasion, cachexia, and morbidity due to obstruction of upper aerodigestive tract, and high incidence of local and distant metastasis.
**MATERIALS AND METHODS**

**Cell Lines and Culture Conditions.** The ATC cell line ARO, DRO, C643, and KAT-4 were used. ARO and DRO cell lines were generated in the laboratory of Dr. G.F.J. Julliard (University of California, Los Angeles, CA). KAT-4 cell line was generated in the laboratory of Dr. K.B. Ain (University of Kentucky Medical Center). C643 cell line was generated in the laboratory of Dr. N.E. Heldin (University Hospital, Uppsala, Sweden). All the cell lines were cultured in primary tumors. As all ATCs are staged as stage IV regardless of size or metastatic status, all the ATC cell lines were generated from stage IV tumors. In addition, we used human squamous cell carcinoma of the oral cavity cell line TU167, which was obtained from the laboratory of Dr. Gary Clayman at the University of Texas M.D. Anderson Cancer Center. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, sodium pyruvate, and nonessential amino acids. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were maintained free of *Mycoplasma* species and were maintained no longer than 12 weeks after recovery from frozen stocks. Before injection into nude mice, the cells were trypsinized and resuspended in serum-free RPMI 1640 at appropriate concentrations.

**Animal Care and Injection.** Eight- to 12-week-old male athymic nude mice 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 done in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Before injection of tumor cell suspension, the mice were first anesthetized with i.p. injection of sodium pentobarbital (50 mg/kg). Cells (5 \times 10^3) from ARO, DRO, and C643 cell lines were suspended in RPMI 1640 at a final dilution of 1 \times 10^5 cells/μL, and 5 μL (5 × 10^3 cells) of that suspension was injected into the thyroid glands of each mouse. Each cell line was injected into five mice. These mice were killed by CO₂ inhalation 2 weeks after the injections, and necropsy was done to examine for the presence of tumors.

After confirming that injection of thyroid carcinoma cell lines into the thyroid gland of nude mice was technically feasible and resulted in tumor development, we specifically examined the tumorigenicity of the ATC cell line DRO and squamous cell carcinoma of the oral cavity cell line TU167 in the thyroid gland and the subcutis. DRO and TU167 cells were injected into the thyroid gland and the subcutis of the right flank at the following concentrations: 5 \times 10^3, 1 \times 10^3, 2.5 \times 10^3, 5 \times 10^3, and 10 \times 10^3 cells. The injection volume was 5 μL (5 × 10^3 cells) for each mouse. A group of five mice were injected for each dose level. The mice were examined for the presence of tumor every other day by manual palpation and gross visual inspection. The presence of tumor was verified by necropsy and by histologic confirmation. The weights of the mice were measured twice a week. The mice were sacrificed if moribund or for weight loss of >20% of their preinjection body weight. Otherwise, the mice were sacrificed 6 weeks after tumor cells injection by CO₂ inhalation. All mice underwent necropsy regardless of whether they were killed at the end of the study or during the study.

**Orthotopic Thyroid Injection Technique.** The entire injection procedure was done with the aid of a dissecting microscope. After administration of sodium pentobarbital, a midline cervical incision was made. The underlying submandibular glands were retracted laterally and the central compartment of the neck was visualized (Fig. 1A and B). Gentle retraction of the midline strap muscles revealed the thyroid gland adjacent to the trachea and visible underneath a deeper layer of semitransparent strap muscles (Fig. 1C). Direct injection of the right thyroid gland was then done using a 25 μL Hamilton syringe (Hamilton Company, Reno, NV) and 30-gauge hypodermic needle. The injection volume was 5 μL. The submandibular glands were returned to the original location and the skin was closed in a single layer with the use of staples.

**Subcutaneous Injection Technique.** Each mouse underwent s.c. injection of cells (suspended in a volume of 5 μL) directly into the flank using a 25-μL Hamilton syringe (Hamilton Company) and 30-gauge hypodermic needle. The needle tip was inserted under the skin with the bevel turned upward, and the cells were injected slowly to prevent tumor shearing.

**Necropsy and Tissue Preparation.** After the mice were killed, the cervical lymph nodes, lungs, and thyroid gland in continuity with the larynx and trachea were removed and placed in formalin solution overnight. Each specimen was embedded in paraffin and then sectioned. The sections were stained with H&E and evaluated by light microscopy for the presence of regional or distant metastasis.

**Immunohistochemical Staining on Tumor Tissues.** To determine the intratumoral microvessel density, staining with antibodies against tumor endothelium was done using rat antimouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (PharMingen, San Diego, CA). Staining for interleukin-8 (IL-8) was done using rabbit anti IL-8 antibody (Biosource, Camarillo, CA).

For CD31/platelet/endothelial cell adhesion molecule 1 and IL-8 staining, fresh frozen tumors were sectioned (8-10 mm thick), mounted on positively charged Superfrost slides (Fisher Scientific, Houston, TX), air-dried for 30 minutes, and fixed in cold acetone for 10 minutes. The slides were washed thrice with PBS (pH 7.5), blocked for 20 minutes at room temperature in PBS supplemented with 1% normal goat serum and 5% normal horse serum (protein-blocking solution), and incubated with primary antibody (diluted 1:800 for CD31/platelet/ endothelial cell adhesion molecule 1 and 1:50 for IL-8) for 18 hours at 4°C. Samples were then washed thrice for 3 minutes with PBS and blocked with protein-blocking solution for 10 minutes, and then incubated with goat anti-rat IgG conjugated to hors eradish peroxidase (Molecular Probes, Eugene, OR) diluted at 1:400 or goat anti-rabbit horseradish peroxidase (Molecular Probes) diluted at 1:400 for 1 hour at room temperature. The slides were washed again in PBS thrice followed by incubation with 3,3’-diaminobenzidine for 10 minutes. The slides stained for IL-8 were counterstained with Gill’s hematoxylin 3 after the degree of staining was quantified as outlined below. The slides stained for CD31/ platelet/endothelial cell adhesion molecule 1 were counterstained with Gill’s hematoxylin 3 directly after incubation in 3,3’-diaminobenzidine.
Fig. 1  A, central compartment of the neck of a nude, athymic mouse (original magnification, ×1). B, removal of the strap muscles exposes the underlying trachea and the thyroid glands (*) which consist of two distinct lobes without an isthmus (original magnification, ×1.6). C, visualization of the thyroid gland (*) through the semitransparent layer of strap muscles before injection (original magnification, ×1.25).
To stain for basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), paraffin-embedded sections were first dewaxed in xylene. Excess xylene was removed by washing the slides in ETOH. After treating the tissue with pepsin for 20 minutes at 37°C, the slides were washed thrice with PBS. Endogenous blocking was done with 3% H2O2 followed by protein blocking using 5% horse serum with 1% goat serum (protein blocking solution). Rabbit anti-human VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 and rabbit anti-human bFGF antibody (Sigma, St. Louis, MI) diluted 1:1,000 were added to the slides for 18 hours at 4°C. The slides were then washed with PBS thrice, blocked again with protein blocking solution for 1 hour, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 dilutions for 1 hour at room temperature. The slides were washed again in PBS thrice followed by incubation with 3,3'-diaminobenzidine for 10 minutes. The degree of staining was then quantified for each slide as outlined below. After quantification of staining, the slides were counterstained with Gill’s hematoxylin 3.

Quantification of Basic Fibroblast Growth Factor, Vascular Endothelial Growth Factor, and Interleukin-8 Staining Intensity and Determination of Microvessel Density. For quantification of the immunohistochemical reaction intensity, the absorbance of bFGF-, VEGF-, and IL-8-positive cells from either orthotopically established or ectopically established ATC tumors were measured in random 0.039 mm² fields at ×100 magnification using the Optimas Image Analysis software (Silver Spring, MD). Five tissue sections were stained for each group and four fields were evaluated for each section. The samples were not counterstained, so that the absorbance was attributable solely to the product of immunohistochemical reaction. To quantify the microvessel density, four 0.159 mm² fields at ×100 magnification were captured for each tumor section, and CD31-positive microvessels were quantified according to methods described previously (14, 15). Five tissue sections were stained for each group and four fields were evaluated for each section.

Microscopy. Stained sections were examined in a Microphot-FX microscope (Nikon, Melville, NY) equipped with a three-chip-charged couple device color video camera (Model DXC990, Sony Co., Tokyo, Japan). The photomontages were prepared using Photoshop software (Adobe Systems, Inc., San Jose, CA).

Statistical Analysis. Because of the relatively small sample size of these experiments and the pattern of observed tumor growth, statistical methods based on standard maximum likelihood based methods were not applicable. As an alternative, penalized logistic regression was used to assess the effects of thyroid versus s.c. inoculation (whereas accounting for the effects of the cell dilution) on tumorigenicity. The regression model included terms for the dilution effect and for the inoculation location (thyroid versus s.c.). Differences in survival times were assessed using the log-rank test. Group differences for continuous measurements were assessed via the nonparametric Kruskal-Wallis test or the nonparametric Wilcoxon rank sum test. Ps for the location effect were evaluated against a α significance level of 0.05. Statistical analysis for comparing microvessel density, bFGF, VEGF, and IL-8 staining between orthotopic and ectopic ATC tumors was done with Student’s t test using SPSS software.

RESULTS

Human Thyroid Carcinoma Cell Lines Formed Tumors when Injected into the Thyroid Glands of Nude Mice. ATC cell lines ARO, DRO, C643, and KAT-4 were injected into the thyroid glands of nude mice at 5 × 10⁵ cells per animal. When examined by necropsy 2 weeks after the injections, all of the cell lines showed 100% tumorigenicity. On histologic examination, infiltration of the tumor into the thyroid gland with interdigitation of the tumor between the thyroid follicles was evident with compression of the thyroid gland against the trachea (Fig. 2A and B).

Tumorigenicity of the Anaplastic Thyroid Carcinoma Cell Line DRO in Orthotopic and Ectopic Organs. After confirming that injection of thyroid carcinoma cell lines into the thyroid gland of nude mice was technically feasible and that it resulted in tumor development, we specifically examined the tumorigenicity of ATC cell line DRO in orthotopic and ectopic locations. This cell line was selected because of its short doubling time as well as relatively high tumorigenicity in nude mice. When injected into thyroid glands of nude mice at concentrations of 5 × 10⁵, 1 × 10⁵,
Fig. 3 Growth of the thyroid tumors (arrows) after injection of ATC cell line DRO into right thyroid gland. The normal left thyroid gland is marked by an asterisk (A) 4 days post injection, (B) 11 days post injection, and (C) 16 days post injection. Original magnification, ×1.25.
2.5 \times 10^4, 5 \times 10^3, \text{ and } 1 \times 10^3 \text{ cells per animal, DRO cell line was extremely tumorigenic, and thyroid tumors developed in 100% of the animals. Even injections of as few as } 1 \times 10^3 \text{ cells resulted in 100% tumorigenicity. In contrast, the tumorigenicity of DRO cell line was significantly lower when injected s.c. S.c. tumors developed in five of five animals inoculated at } 5 \times 10^5 \text{ cells per animal, three of five animals inoculated at } 1 \times 10^5 \text{ cells per animal, two of five animals inoculated at } 2.5 \times 10^4 \text{ cells per animal, one of five animals inoculated at } 5 \times 10^3 \text{ cells per animal, and none developed in the animals inoculated with } 1 \times 10^3 \text{ cells. Analysis by the penalized logistic regression model showed that the difference in tumorigenicity observed between those mice inoculated s.c. (ectopic) and those mice given thyroid inoculations (orthotopic) was statistically significant (} P < 0.0001\text{). These data show that DRO cells were significantly more tumorigenic at the orthotopic site than at the ectopic, s.c. site.}

**Tumorigenicity of the Squamous Cell Carcinoma of the Oral Cavity Cell Line TU167 when Injected into the Thyroid Gland and Subcutis of Nude Mice.** It is possible that the thyroid gland of nude mice is generally more permissive to tumor formation than the subcutis, regardless of the organ of origin of the injected tumor cell line. It is also possible that the local injury introduced during the thyroid injection procedure may create an environment more permissive to tumor formation. To assess this possibility, the squamous cell carcinoma of the oral cavity cell line, TU167, was injected into the thyroid glands and the subcutis of nude mice at } 5 \times 10^5, 1 \times 10^5, 2.5 \times 10^4, 5 \times 10^3, \text{ and } 1 \times 10^3 \text{ cells per animal. It has been shown previously that TU167 efficiently forms tumors when injected at the above range of concentrations into its orthotopic site (tongue) in nude mice (16). However, none of the animals inoculated s.c. with TU167 developed tumors, regardless of the concentration of injected cells. More importantly, the tumorigenicity of TU167 was only marginally higher in the thyroid gland where tumors developed in only two of five animals inoculated with } 5 \times 10^3 \text{ cells per animal. In contrast to the DRO cell line, the difference in tumorigenicity for TU167 in the thyroid gland and the subcutis was not statistically significant (} P = 0.18\text{).}

**Orthotopic Model of Anaplastic Thyroid Carcinoma in Nude Mice Recapitulates the Clinical Features of Human Anaplastic Thyroid Carcinoma.** One of the striking features of the ATC is its rapid and invasive growth at the primary site. Animals inoculated with DRO cell line showed rapid tumor growth causing tracheal and esophageal compression (Fig. 3). The mice became moribund and cachetic within 1 to 2 weeks following the injection. At the time of necropsy, compression of the trachea and esophagus were confirmed. The differences in survival duration of the animals were directly related to the concentration of the injected cells (Fig. 4) and were statistically significant.

**Fig. 4** Kaplan-Meier survival curve for the groups injected with different tumor cell concentration into the thyroid gland. The Ps < 0.05 for all groups except animals injected with } 5 \times 10^3 \text{ cells per animal when compared with the group injected with } 1 \times 10^3 \text{ cells per animal.}

**Fig. 5** A, axial section of the larynx showing tumor (*) invasion of the paraglotic space via erosion through the inferior constrictor muscles posterior to the thyroid cartilage. B, tracheal invasion evident by the nest of tumor cells (*) interpositioned between the tracheal mucosa and cartilage. C, pulmonary metastasis. D, cervical lymph node with subcapsular metastatic tumor. (A–D: H&E stain; original magnification, ×40).
The presence of metastatic disease.

Nodes as well as the lungs were removed and examined histologically for

otherwise, the mice were sacrificed at the end of 6-week period. All of

nude mice at the indicated concentrations. Five mice were injected per

group. The mice were sacrificed if moribund or for weight loss >20%.

otherwise, the mice were sacrificed at the end of 6-week period. The

thyroid glands in continuity with the laryngotraechae were removed and

examined histologically for the presence of tumor invasion.

significant. The mean survival durations were 18, 26, and 31
days for animals injected with 5 × 10^5, 1 × 10^5, and 2.5 × 10^4
cells per animal, respectively.

Frequent direct invasion of the adjacent structures such as the

larynx and trachea is another hallmark of ATC. At the time of

necropsy, the thyroid tumors were resected in continuity with the

larynx and trachea, embedded in paraffin, and examined for

invasion of the larynx or trachea by the tumor cells. Laryngeal

invasion was present in several animals and involved the para-

glottic space (Table 1; Fig. 5A). Tumor invasion into the

paraglottic space seemed to occur most commonly through the

inferior constrictor muscle posterior to the thyroid cartilage

(Fig. 5A). Although less frequent than laryngeal invasion,

tracheal invasion was also evident in these animals (Fig. 5B).

these data showed that the orthotopic model of ATC in nude

mice reproduced the clinical and pathologic features that are

characteristic of human ATC.

Orthotopic Model of Anaplastic Thyroid Carcinoma in

Nude Mice Recapitulates the Metastatic Pattern of Human

Anaplastic Thyroid Carcinoma. It has been noted that up to

70% patients with ATC will develop systemic metastasis, with

the lung being the most common site, accounting for up to 80%
of cases (17). Likewise, mice injected with the DRO cell line

exhibited a high rate of pulmonary metastasis (Fig. 5C; Table 2).

The highest rate (80%) of pulmonary metastasis was noted in

animals inoculated with 2.5 × 10^4 cells per animal. The lower

incidence of pulmonary metastasis in animals inoculated with a

higher cell concentration seems to be due to the short life span of

these groups of animals. Metastatic disease in the cervical lymph

nodes was also evident in these animals although at lower rates

(Fig. 5D).

Levels of Vascular Endothelial Growth Factor and

Interleukin-8 Expressions and Microvessel Density Are

Higher in Orthotopic Thyroid Tumors than in Ectopic

Thyroid Tumors. To show organ-specific interactions between

the tumor and the host tissue that are not present in ectopic sites,

we compared the microvessel density of orthotopic and ectopic

thyroid tumors. Microvessel density was significantly higher in

orthotopic thyroid tumors than in ectopic thyroid tumors

established in the subcutis (P < 0.05; Fig. 5; Table 3). Immunohistochemical staining for proangiogenic factors VEGF

and IL-8 showed that these factors were also expressed at higher

levels in the orthotopic tumors than in ectopic thyroid tumors

(P < 0.05; Fig. 5; Table 3). The difference in the intensity of

staining for bFGF was not statistically significant between the

orthotopic and ectopic thyroid tumors (P = 0.241).

DISCUSSION

The development and evaluation of novel anticancer agents require the use of an appropriate animal model, which

can accurately recapitulate the disease process (5, 6). Although s.c. xenograft models of cancer in nude mice are

often used in preclinical studies, these models are problematic in several aspects. The s.c. xenograft model lacks the

specific interactions that exist between the tumor cells and their native environment that influences the molecular,

pathologic, and clinical features of the tumor (5–11). Establishment of tumor at the orthotopic site restores these distinct

patterns of interactions between the tumor and the host organs that are lost or altered when the tumors are established in ectopic

sites (5, 6, 8–11).

The site-specific interaction between the tumor cells and the

host organ has been shown to influence the phenotype of tumor

cells in various aspects. It has been shown previously that

sensitivity to chemotherapeutic drugs differs between orthotopi-

cally established tumors and the same tumor established
ectopically (10, 11). Furthermore, the interactions between the

tumor cells and the host organs influence the expression of

autocrine and paracrine growth factors as well as their receptors

(8, 11). In this study, ATC cell line DRO showed significantly

higher tumorigenicity when injected into the thyroid gland, and the

orthotopic tumors showed higher microvessel density than


**Table 1** Local invasive potential of DRO cell line when injected into the thyroid glands of nude mice

<table>
<thead>
<tr>
<th>DRO (no. cells injected/mouse)</th>
<th>% Mice with laryngeal invasion</th>
<th>% Mice with tracheal invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10^3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5 × 10^4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 × 10^5</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>5 × 10^5</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

NOTE. ATC cell line DRO was injected into the thyroid glands of nude mice at the indicated concentrations. Five mice were injected per group. The mice were sacrificed if moribund or for weight loss >20%. Otherwise, the mice were sacrificed at the end of 6-week period. All of the mice underwent necropsy. At the time of necropsy, the cervical lymph nodes as well as the lungs were removed and examined histologically for the presence of metastatic disease.

**Table 2** Metastatic potential of ATC cell line DRO when injected into the thyroid gland of nude mice

<table>
<thead>
<tr>
<th>DRO (no. cells injected/mouse)</th>
<th>% Mice with pulmonary metastasis</th>
<th>% Mice with cervical lymphatic metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10^3</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2.5 × 10^4</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>1 × 10^5</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>5 × 10^5</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. ATC cell line DRO was injected into the thyroid glands of nude mice at the indicated concentrations. Five mice were injected per group. The mice were sacrificed if moribund or for weight loss >20%. Otherwise, the mice were sacrificed at the end of 6-week period. All of the mice underwent necropsy. At the time of necropsy, the cervical lymph nodes as well as the lungs were removed and examined histologically for the presence of metastatic disease.

**Table 3** IHC analysis of orthotopic and ectopic thyroid tumors

<table>
<thead>
<tr>
<th></th>
<th>MVD*†</th>
<th>IL-8†</th>
<th>VEGF</th>
<th>bFGF§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthotopic thyroid tumors</td>
<td>17.8 ± 6.2</td>
<td>2.01 ± 0.51</td>
<td>0.44 ± 0.20</td>
<td>0.41 ± 0.12</td>
</tr>
<tr>
<td>Ectopic thyroid tumors</td>
<td>9.6 ± 3.8</td>
<td>0.80 ± 0.36</td>
<td>0.31 ± 0.12</td>
<td>0.47 ± 0.22</td>
</tr>
</tbody>
</table>

*Mean ± SD positive cells/field determined from measurement of 20 random 0.159 mm² fields at ×100 magnification.

†P < 0.05 between orthotopic and ectopic tumor groups.

‡Mean ± SD absorbance determined as described in Materials and Methods.

§P = 0.241 between orthotopic and ectopic tumor groups.
ectopically established ATC tumors. This increase in microvessel density was accompanied by a corresponding increase in tumor production of VEGF and IL-8. These cellular and molecular differences between orthotopic and ectopic ATC tumors most likely represent the restoration of tumor-host organ interaction that is lost at the ectopic sites.

The major utility of orthotopic cancer models is in recapitulation of the metastatic potentials and patterns seen in human cancer. This requirement of an orthotopic model in promoting metastasis arises from both anatomic and biological constraints. First, the lymphatic drainage and the vascular supply of the subcutis are vastly different than the orthotopic organ. Therefore, the s.c. xenograft model cannot reproduce the patterns of regional and distant metastasis that are characteristic of a particular human cancer. Second, the biological differences between the orthotopic and ectopic location also contribute to the extremely low metastatic rates of s.c. established tumors. Therefore, highly metastatic cancer models have been established in nude mice for carcinomas of the bladder, stomach, pancreas, lung, and the oral tongue by inducing these tumors orthotopically (12, 13, 16, 18, 19). Likewise, our orthotopic model of ATC produced cervical lymphatic metastasis within the regional draining lymphatic basin. This model also produced high rates of pulmonary metastasis that are reflective of human ATC.

Another advantage of an orthotopic model of cancer is its ability to reproduce the site-specific spectrum of clinical findings and the effects these findings have on survival. As seen in patients with ATC, orthotopically produced ATC in nude mice produced high incidence of tracheal and esophageal compression as well as direct laryngeal/tracheal invasion. As result, the animals became moribund and required sacrifice within 3 to 4 weeks after tumor cell injection. Upon necropsy, obstruction of upper aerodigestive tract was confirmed to be the cause of the morbidity. These features of the orthotopic model will allow the evaluation of antitumor compounds in its ability to prevent the local extension of the disease and also to influence survival.

For any animal model to be useful, it must be technically feasible and relatively easy to perform. Injection of the thyroid glands can be done in 2 to 3 minutes per mouse. The procedure was also well tolerated by the animals with post-procedure mortality of <1%. Also, by performing only unilateral injection of the thyroid gland, any iatrogenic morbidity such as hypothyroidism, hypoparathyroidism, or bilateral vocal cord paralysis were prevented.

Lastly, as with any animal model, the limitations of this orthotopic model must be considered. Although the use of orthotopic site is a vast improvement in disease modeling over the use of ectopic sites (s.c.), the biology and physiology of immunodeficient mice does not correlate directly with that of human. Where possible, findings derived from this model should be additionally substantiated with studies in immunocompetent models and analysis of human tumor specimens.

In conclusion, we have developed an orthotopic model of ATC in nude, athymic mice that reproduces the clinical and pathologic features of ATC in human. This orthotopic model will facilitate the development and evaluation of novel therapeutic compounds for the treatment of this almost uniformly fatal disease. Furthermore, the model should allow further progress in enhancing our understanding into the molecular and cellular mechanisms that underlie the pathogenesis and metastasis of ATC.

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