Published in final edited form as:

Radiat Res. 2011 October ; 176(4): 486–493.

A Novel Bioluminescence Orthotopic Mouse Model for Advanced Lung Cancer

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Abstract

Lung cancer is the leading cause of cancer-related death in the United States despite recent advances in our understanding of this challenging disease. An animal model for high-throughput screening of therapeutic agents for advanced lung cancer could help promote the development of more successful treatment interventions. To develop our orthotopic lung cancer model, luciferase-expressing A549 cancer cells were injected into the mediastinum of athymic nude mice. To determine whether the model would allow easy monitoring of response to therapeutic interventions, tumors were treated with 30 mg/kg Paclitaxel or were irradiated with 5 fractions of 2 Gy, and tumor burden was monitored using bioluminescence imaging. Evidence of radiation-induced lung injury was assessed using immunohistochemical staining for phospho-Smad2/3 and cleaved caspase-3. We found that tumor implantation recapitulated advanced human lung cancer as evidenced by tumor establishment and proliferation within the mediastinum. The tumor responded to Paclitaxel or radiation as shown by decreased tumor bioluminescence and improved overall survival. Immunohistochemistry revealed increased phospho-Smad2/3 and cleaved caspase-3 in irradiated lungs, consistent with radiation-induced lung injury. This orthotopic lung cancer model may help provide a method to assess therapeutic interventions in a preclinical setting that recapitulates locally advanced lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the United States, indicating an important need for improvements in treatment outcomes. Development of reliable mouse lung cancer models representative of human disease would allow preclinical screening of new therapies to help control this disease.

Most preclinical testing of lung cancer therapies use ectopic cancer models created by subcutaneous implantation of human lung cancer cells into readily accessible body sites, including thighs or shoulders (1, 2). However, the ease of tumor size measurement in these contained models comes at the expense of creating a different microenvironment from that of naturally occurring lung cancer. This may pose questions of clinical translation because tumor development depends on the tissue surroundings (3), and inconsistencies in tumor response to chemotherapy has been demonstrated between ectopic and orthotopic cancer models (4). Furthermore, radiotherapy of ectopic tumor models may not accurately reflect the concurrent damage of healthy lung tissue. Thus orthotopic animal models where human

cancer cells grow in a microenvironment more akin to human lung tissue are necessary for preclinical drug testing.

To date, a number of orthotopic lung cancer models have been developed. Introduction of Lewis lung carcinoma cells into the lung parenchyma by direct puncture without thoracotomy has demonstrated establishment of a solitary intrapulmonary nodule with some lymph node metastasis (5). Similarly, a previous study of intratracheal injection of A549 cancer cells has shown establishment of a large primary tumor with lymph node involvement (6). These models may be well suited for studying early-stage lung cancer. However, since most lung cancer patients are diagnosed after the development of advanced, inoperable disease (7, 8), an animal model representing bulky mediastinal disease reflective of advanced human lung cancer is necessary. Some orthotopic models have successfully demonstrated widespread lung tumor penetration, but euthanizing mice with subsequent thoracotomy was required to quantify changes in tumor growth and spread (9). Similarly, previously proposed endobronchially implanted models have demonstrated successful tumor growth and extrapulmonary metastases (10). However, in addition to a more difficult technical barrier, compared to our proposed model, this model also required that mice be euthanized to evaluate tumor growth. Therefore, development of an orthotopic lung cancer mouse model with locally advanced disease and bulky mediastinal tumor involvement with a simple, noninvasive method for tumor growth detection remains a significant challenge.

In this paper, we describe an orthotopic lung cancer model established by mediastinal injection of A549 cancer cells transfected with luciferase (A549-Luc cells). Luciferase-expressing cells allow noninvasive tumor visualization by bioluminescence imaging. We found that this lung cancer model responds to known effective treatment interventions including Paclitaxel and radiotherapy, and tumor burden could easily be monitored with bioluminescence in the same animal over time. Furthermore, we were able to directly observe radiation-induced lung injury. This model could allow for the investigation of novel agents in a preclinical setting reflective of advanced lung cancer. In addition, this model will possibly allow novel combination treatment (new agent and radiation) testing with simultaneous monitoring of lung toxicity.

MATERIALS AND METHODS

Cell Line

Human lung adenocarcinoma cells (A549) were obtained from American Type Culture Collection (ATCC, Manassas, VA). A549-Luc cells, which have previously been validated as stable clones, were obtained from Dr. Jerry W. Shay (University of Texas Southwestern, Department of Cell Biology, Dallas). These A549-Luc cells were derived from A549 cells as described by Dikmen et al. (11). In brief, the A549-Luc cell line was generated using a lentivirus, which encodes the luciferase gene driven by a ubiquitin promoter. The lentivirus was produced in 293T cells; A549 cells were then infected with the virus-containing medium. After infection, fresh F12 medium was added to allow cells to recover for 24 to 48 h. The cells were then selected using G418 (500 µg/ml) for 2 weeks in 10-cm dishes with a cell density of 3×10^4 . Cells were grown in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Both cell lines were kept at 37°C with humidified 95% air/5% CO₂.

Reagents, Treatments and Antibodies

D-Luciferin Firefly and potassium salt were purchased from Gold Biotechnology (St. Louis, MO) and dissolved in DPBS (15 mg/ml). The solution was filter sterilized through a 0.2-µm filter. Each mouse was injected at a dose of 100 mg/kg intraperitoneally 15 min prior to

imaging. Paclitaxel was purchased from Sigma-Aldrich (catalog number T1912) and dissolved in DMSO (50 mg/ml). Irradiation was performed using an X irradiator (Therapax, AGFANDT, Inc., Lewistown, PA). Anti-phospho-smad2/3 (Ser423/425) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-caspase 3 was purchased from Cell signaling Technology (Danvers, MA).

Development of Orthotopic Lung Cancer Model

Forty 5–6-week-old female athymic nude mice (nu/nu) were purchased from Harlan Sprague Dawley Inc. Mice were anesthetized by continuous flow of 2–3% isoflurane. The chest area was cleaned with a 70% alcohol pad three times followed by air drying for 10 s. A549-Luc cells (3×10^6) suspended in 100 μ l of DPBS were injected into the mediastinum (Fig. 1A). The needle (1 ml, 26G3/8 from BD) was inserted 5 mm into the right side of and midway down the sternum at an angle of approximately 45° with the sternum.

Tumor Assessment by Bioluminescence

When tumor size reached a level detectable by bioluminescence (in our case, 5×10^4 photon counts), mice with similar photon counts were selected and assigned to either the control group or treatment group. Bioluminescence was measured on the Genogen IVIS™ 200 system starting on day 5 of implantation. Photon counts for each group were averaged; the average derived from the control group on the first day of treatment was arbitrarily defined as the base level. In the subsequent bioluminescence scans, photon counts were converted to relative levels compared to the base level.

Tumor Histology

Tumors in mice were removed, fixed in 10% formalin for 24 h, and processed and stained with H&E at Vanderbilt University's immunohistochemistry core. Tumor samples were analyzed for histological changes including the presence of tumor necrosis, apoptosis, mitotic activity and cytologic atypia.

Tumor Growth Response to Paclitaxel and Radiation Therapy

All animal procedures used in these studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University, and the housing and handling of animals followed IACUC guidelines. For studies using Paclitaxel, 10 mice with similar photon counts (minimum threshold 5×10^4 photon counts on 2 consecutive days) of 20 mice implanted were stratified into two treatment groups ($n = 5$). Mice received 30 mg/kg Paclitaxel or DMSO control i.p. on days 1, 5 and 9. For studies using radiation, 10 mice with similar photon counts (minimum threshold 5×10^4 photon counts on 2 consecutive days) of 20 mice implanted were stratified into two treatment groups ($n = 5$). The treatment group was irradiated with 2-Gy fractions and the control was sham-irradiated for 5 five consecutive days for a total dose of 10 Gy. A Pantak X-ray source was used, operating at 300 kVp and 10 mA. Ionizing radiation was applied to the thorax; the area of irradiated tissue was approximately 2–2.5 cm². The rest of the body above the base of neck and below the xyphoid process was shielded from radiation using lead blocks. Tumor growth and volume were assessed using the Xenogen IVIS-200 bioluminescence system starting on day 8. The *D*-Luciferin Firefly/potassium salt solution (150 mg/kg) was injected 15 min prior to each bioluminescence scan. For consistent analysis during follow-up scans, exposure time was set at 5 s.

Lung Immunohistochemistry

One mouse from the radiation group and one mouse from the corresponding control group were euthanized 2 weeks after the first dose of radiation with subsequent removal of the left

lungs, fixation and embedding in paraffin. Paraffin-embedded lung tissue slides were deparaffinized by 100% xylene, rehydrated through gradient ethanol series, and washed with PBS. The antigen was retrieved by boiling slides in citrate for 10 min and quenching endogenous horseradish peroxidase (HRP) activity by treating slides with 3% hydrogen peroxide for 30 min. Antibodies to phospho-Smad2/3 (1:20) and cleaved caspase 3 (1:100) were added to sections. Slides were placed in a moist container and refrigerated overnight at 4°C. The next day, slides were washed three times with PBS, and secondary antibody conjugated to HRP (1:100) was added for 1 h. Slides were washed with PBS followed by addition of DAB substrate for 2–5 min. Slides were washed with water to stop the reaction and counterstained with hematoxylin for 1 min. Slides were dehydrated and mounted with cover slips. Positive cells were identified visually. The percentage of positive cells was calculated by dividing the positive cells by the total cells in each high-power field (40× magnification). A total of 10 fields were observed.

Statistics

The Mann-Whitney *U* test was used to provide statistical analysis. Normal distribution was not assumed.

RESULTS

Development of Lung Cancer in the Orthotopic Mouse Model

To develop the orthotopic lung cancer model, A549-Luc cells (3×10^6) expressing luciferase were injected into the mediastinum of nude mice (Fig. 1A). Bioluminescence showed baseline luciferase activity after initial injection in the mediastinum and lungs of all mice, indicating effective tumor transplantation. Over 17 days, the area and intensity of bioluminescence activity steadily increased (Fig. 1B). Gross nodular tumor burden was evident in lungs and mediastinum when mice were dissected after natural death (data not shown). Microscopic examination of tumor tissue demonstrated a moderately differentiated adenocarcinoma with glandular pattern and intracellular mucin, consistent with successful A549 tumor implantation (Fig. 1C). Focal areas of early necrosis were seen. Apoptotic cells were prominent both around and at a distance from the necrotic areas (approximately 20 apoptotic bodies per 10 high-power fields). Cells undergoing mitosis were seen (approximately 5 mitoses per 10 high-power fields). The life span of tumor implanted mice was 12–28 days without therapeutic intervention.

Paclitaxel Suppresses Tumor Growth in the Orthotopic Lung Cancer Mouse Model

To determine whether this orthotopic lung cancer model could allow testing of tumor response to treatment, mice were treated with Paclitaxel since previous studies have shown that A549 cells are sensitive to this drug (12). Mice injected with A549-Luc cells received 30 mg/kg Paclitaxel or DMSO, and tumor growth was followed over time with bioluminescence. Initial bioluminescence activity in both groups was comparable (not significantly different) at baseline. Mice treated with DMSO control showed a steady increase in bioluminescence activity over time, suggesting tumor growth. In contrast, while mice treated with 30 mg/kg Paclitaxel showed an initial increase in bioluminescence activity, on day 9 tumor growth began to subside. By day 13, the relative increase in bioluminescence activity was significantly lower in mice treated with Paclitaxel than in the corresponding controls (Fig. 2A; $P < 0.05$). The peak of bioluminescence activity on day 9 and the subsequent decrease in activity is illustrated in Fig. 2B. Microscopic examination of tumor tissue treated with Paclitaxel demonstrated a moderately differentiated adenocarcinoma without more atypia than the untreated tumor. Approximately 10% tumor necrosis and up to 13 apoptotic bodies per 10 high-power fields were seen. Mitoses were increased over the untreated tumor and numbered up to 14 per 10 high-power fields (Fig.

2C). Moreover, mice treated with Paclitaxel had a significantly higher overall survival rate than corresponding controls (Fig. 2D; $P = 0.012$). While all mice in the DMSO control group died by day 28, 40% of mice treated with Paclitaxel survived longer than 60 days.

Radiation Suppresses Tumor Growth in the Orthotopic Lung Cancer Mouse Model

A549 cells are known to be sensitive to radiation (13). To determine whether this orthotopic lung cancer model responds to radiotherapy and thus might allow the pre-clinical assessment of radiosensitizers, mice injected with A549-Luc cells received 5 fractions of 2 Gy radiation or were sham-irradiated. Bioluminescence activity was followed over time to monitor tumor response to radiation. There was no significant difference in the initial bioluminescence activity at baseline of the two groups. By day 11, mice treated with radiation showed a significantly lower relative increase in bioluminescence activity than corresponding controls (Fig. 3A, $P < 0.05$). Similarly, Fig. 3B shows the lesser increase in bioluminescence activity in irradiated mice relative to control mice (shown in Fig. 1B). Microscopic examination of the irradiated tumor demonstrated a moderately differentiated adeno-carcinoma with nuclear atypia and pleomorphism that were greater than in the untreated tumor. The tumor tissue showed approximately 10% necrosis, prominent apoptosis (up to 23 apoptotic bodies per 10 high-power fields), and up to 7 mitoses per 10 high-power fields (Fig. 3C). Moreover, mice treated with radiation had a significantly higher overall survival rate than corresponding controls (Fig. 3D; $P = 0.004$). Two mice in the untreated group did not survive beyond day 17. All mice in the control group died by day 28, while 20% of mice treated with radiation survived longer than 60 days.

Immunostaining for Radiation-Induced Lung Injury in the Orthotopic Lung Cancer Mouse Model

Radiation-induced lung injury, which consists of radiation pneumonitis and subsequent pulmonary fibrosis, represents the most common and potentially fatal dose-limiting toxicity in patients receiving thoracic radiotherapy (14). TGF- β plays a central role in promoting the development of radiation-induced inflammation and fibrosis. The inflammatory response induced by TGF- β is largely mediated by Smad proteins (15). In addition, TGF- β -induced remodeling and fibrosis sometimes requires caspase-dependent activation of apoptosis (16). Therefore, we used phospho-Smad2/3 (activated form) and cleaved caspase-3 as markers for radiation-induced lung toxicity. Mice were euthanized 2 weeks after the first dose of radiation, and left lungs were removed for immunostaining. Mice treated with radiation showed increased levels of phospho-Smad2/3 compared to corresponding controls (47.6% and 8.8%, respectively, Fig. 4; $P = 0.0001$). Similarly, irradiated mice showed increased active caspase-3 staining compared to controls (49.0% and 6.8%, respectively, Fig. 4; $P = 0.002$). This suggests that irradiation of lungs induced TGF- β -mediated changes consistent with radiation-induced lung injury.

DISCUSSION

Previous generations of orthotopic lung cancer models have had limitations regarding the extent of tumor metastasis, resulting in animal models more suitable for studying earlier-stage lung cancer (5, 6, 9). In this paper, we describe an orthotopic lung cancer model using A549-Luc cells injected into the mediastinum of nude mice. This strategy has low technical barrier and a high success rate (40/40 mice) of tumor transplantation and recapitulates advanced lung cancer throughout the mediastinum. This model is important since most lung cancer patients present with advanced inoperable lung cancer at diagnosis (8).

All mice showed reliable tumor implantation with only minor variation in initial tumor burden. Increased SEM observed in the untreated group likely reflects a constant signal-to-

noise ratio inherent to bioluminescence. Noninvasive estimation of tumor volume with bioluminescence allowed rapid *in vivo* tracking of tumor growth. Other orthotopic lung cancer models have used green fluorescent protein (GFP) imaging (6, 8). Red fluorescent protein (RFP) imaging has also been used in other orthotopic models, most notably for pancreatic cancer (17). However, bioluminescence is more sensitive and quantitative than either, providing the capability to observe bioluminescence signals at the time of tumor implantation and quantify tumors that are not grossly measurable (18). Furthermore, bioluminescence is able to illustrate the number of metabolically active tumor cells, allowing for a better evaluation of treatment efficacy on tumor physiology as opposed to other methods that provide only a volumetric measurement of tumor mass, such as MRI (19). PET scanning holds some advantages over bioluminescence; specifically, its signal is not as limited by depth and it is better suited for 3D localization of tumors (20). However, given the low cost, high sensitivity and high-throughput capability of bioluminescence, it is a preferred alternative to PET for cutting the cost of and accelerating the analysis of drug efficacy in an orthotopic mouse model. The usefulness of bioluminescence was demonstrated in our experiments, showing easy monitoring of tumor response to Paclitaxel and to 10 Gy radiation. In response to both treatment modalities, mice exhibited measurable reductions in tumor bioluminescence over time that correlated with improvements in overall survival. The same strategy could be applied to other drugs or combinations of chemotherapy and radiation therapy to assess preclinical efficacy.

Patients with advanced non-small cell lung cancer (NSCLC) treated with radiation only have a 1-year survival of 40–46% (21, 22). When treated with Paclitaxel as a single modality, patients with stage IV NSCLC have a 1-year survival of 38–42% (23). Tumor response to treatment is evident in either case, and we aimed to mimic such a response in our model. This study was not designed to compare overall survival with the two treatments, although we did observe 60-day survivals of 40% and 20% for Paclitaxel and radiation, respectively. While it is difficult to compare the 60-day study of survival in mice to 1-year survival in humans, our model demonstrated a response to clinically established treatments. The ability to replicate increased survival with established treatments demonstrates that tumor physiology in this mouse model can at least partially mirror tumor physiology in humans.

Although our experiments were not optimized for evaluation of radiation-induced lung injury and only minimal histopathological changes were observed in irradiated lung parenchyma (data not shown), we still established proof of concept that lung toxicity from radiotherapy can be measured. Measurement of radiation-induced lung injury is important since it represents the most common dose-limiting toxicity to radiotherapy in the lungs. Detection of early molecular biomarkers associated with radiation-induced lung injury such as TGF- β 1, phosphorylation status of its downstream modulator Smad2/3, or proinflammatory cytokines such as IL-1, IL-6 and IL-8 represent useful alternatives to histopathology (14, 24, 25). In our orthotopic lung model, phospho-Smad 2/3 and cleaved caspase-3, which help mediate TGF- β -induced inflammation in response to radiation, were significantly higher in irradiated lungs than in corresponding controls. Since normal lung parenchyma exhibited measurable markers of toxicity to radiotherapy, future studies could potentially use this technique to investigate radioprotectors and for normal lung tissue. Also, combination treatments aimed at increasing the therapeutic ratio of radiation treatment will be able to monitor lung toxicity to treatment in the preclinical setting.

It is possible that our model lacked significant radiation-induced histopathological changes because the athymic nude mice used in this study are less susceptible to radiation-induced lung injury than sensitive strains such as C57BL/6 mice. Also, the onset of pneumonitis, either acute or delayed, requires at least 3–4 months post-treatment to observe significant

lung injury (26). To better investigate radiation-induced lung injury in our orthotopic lung cancer model, use of more susceptible mouse strains with longer follow-up may be required.

In conclusion, we describe an orthotopic lung cancer model with low technical barrier that allows noninvasive monitoring of tumor response to treatments including Paclitaxel and radiotherapy by bioluminescence. Evaluation of radiation-induced lung injury after irradiation of lungs can be performed using immunohistochemistry. Thus this model could provide a valuable means to evaluate cytotoxic treatment agents, radiosensitizers and normal tissue radioprotectors in a preclinical setting that recapitulates locally advanced lung cancer. Such studies with this model may lead to increased effectiveness of radiation therapy in the presence of diminished radiation-induced lung injury.

Acknowledgments

This work is supported by NCI IR01 CA125842-01A1.

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FIG. 1.

Tumor implantation, volume assessment with bioluminescence, and tumor histology. Nude mice were injected with A549-Luc cancer cells on the right side of the sternum (lower arrow), angled at 45° toward the spine. The needle (point of arrowhead) was inserted about 5 mm in depth. Shown is a schematic diagram (panel A) of the orthotopic lung cancer model. The orthotopic tumor growth within the mediastinum was followed with bioluminescence imaging for 17 days. The intensity of bioluminescence is represented by the color (panel B). H&E-stained paraffin-embedded tumor samples (panel C) were analyzed for the presence of tumor necrosis, apoptosis, mitotic activity and degree of cytologic atypia. An image of the gross tumor removed from the mediastinum is shown in the upper left corner.

FIG. 2.

Tumor response to Paclitaxel. A549-Luc cells were implanted into the mediastinum of nude mice. Mice were treated with 30 mg/kg Paclitaxel (panel A) or DMSO control on days 1, 5 and 9. Relative increase in bioluminescence was calculated as the proportion of bioluminescence to baseline bioluminescence, and SEM is indicated, $*P < 0.05$. The intensity of bioluminescence is represented by the color; the effects of Paclitaxel are evident (panel B). H&E-stained paraffin-embedded tumor samples (panel C) were analyzed for the presence of tumor necrosis, apoptosis, mitotic activity and degree of cytologic atypia. Survival curves for mice treated with 30 mg/kg Paclitaxel (panel D) were followed for 60 days and compared to untreated controls (solid lines). Analysis was performed using a Kaplan-Meier test.

FIG. 3.

Tumor response to radiation therapy. A549-Luc cells were implanted into the mediastinum of nude mice. Mice were treated with 2-Gy fractions of radiation (RT) (panel A) or sham-irradiated for 5 consecutive days. Relative increase in bioluminescence was calculated as the proportion of bioluminescence to baseline bioluminescence; SEM is indicated, $*P < 0.05$. The intensity of bioluminescence is represented by the color; the effect of radiation is evident (panel B). H&E-stained paraffin-embedded tumor samples (panel C) were analyzed for the presence of tumor necrosis, apoptosis, mitotic activity and degree of cytologic atypia. Survival curves for mice treated with radiation (panel D) were followed for 60 days and compared to untreated controls (solid lines). Analysis was performed using a Kaplan-Meier test.

FIG. 4.

Immunostaining for inflammatory mediators of radiation-induced lung injury. Two weeks after mice received the first dose of radiation (RT), lungs of both irradiated and unirradiated mice were removed and immunostained for phospho-Smad2/3 and active caspase-3. Shown are representative photographs of histology slides (panel A). Also shown is a bar graph (panel B) of the mean percentages of cells that stained positive for phospho-Smad2/3 and active caspase-3 with SEM. *P* values are indicated.