Development of an Orthotopic Model to Study the Biology and Therapy of Primary Human Lung Cancer in Nude Mice

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ABSTRACT

Purpose: **This study was conducted to develop biologically relevant animal models of human lung cancer that are reproducible, inexpensive, and easy to perform.**

Experimental Design: **Human lung adenocarcinoma (PC14PE6), bronchioloalveolar carcinoma (NCI-H358), squamous cell carcinoma (NCI-H226), poorly differentiated non-small cell lung cancer (NCI-H1299 and A549), or small cell lung cancer (NCI-H69) cells in Matrigel were injected percutaneously into the left lungs of nude mice. The growth pattern of the different lung cancer tumors was studied. For PC14PE6 and NCI-H358, the growth pattern in the subcutis and the response to paclitaxel were also studied.**

Results: **As is observed for human primary lung cancer, tumors formed from a single focus of disease and progressed to a widespread and fatal thoracic process characterized by diffuse dissemination of lung cancer in both lungs and metastasis to intra- and extrathoracic lymph nodes. When the lung cancer cell lines were implanted s.c., systemic therapy with paclitaxel induced tumor regression. However, only a limited therapeutic response to paclitaxel was observed when the same cells were implanted orthotopically into the lung. Immunohistochemical analysis of tumor tissue revealed increased expression of the proangiogenic factors interleukin 8, basic fibroblast growth factor, and vascular endothelial growth factor/vascular permeability factor.**

Conclusions: **Our orthotopic models of human lung cancer confirm the "seed and soil" concept and likely provide more clinically relevant systems for the study of both non-small cell lung cancer and small cell lung cancer biology, and for characterizing novel therapeutic strategies.**

INTRODUCTION

Lung cancer is a major health problem worldwide, and the leading cause of cancer related death for both men and women in the United States with an annual mortality rate that exceeds breast, prostate, and colorectal cancers combined (1). In most cases, lung cancer patients are diagnosed with advanced inoperable disease, and the only therapeutic option is systemic chemotherapy. Unfortunately, recent studies have concluded that conventional therapies may have reached a ceiling of clinical impact as evidenced by the 5-year survival for NSCLC[4] and SCLC, which remains at 14% and 5%, respectively (2). Clearly, a new approach to the therapy of lung cancer is mandatory. Because organ microenvironment influences the phenotype of tumor cells, as originally enunciated by Pagets' "seed and soil" theory (3) and confirmed by others (4–6), the identification of novel therapeutics depends on the availability of biologically relevant *in vivo* models (7).

NSCLC represents 80% of all lung cancer cases, and most research focuses on this subtype, including the development of several orthotopic models of human NSCLC in rodents. These models include implantation of human cancerous tissue obtained surgically (8) and the injection of tumor cells into the rodent airways (9–11), pleural cavity (12, 13), or lung parenchyma after skin incision (14) or thoracotomy (15–17). In contrast, only two reports describe the use of orthotopic models to study SCLC, which comprises 20% of all lung cancer cases (5, 12). Despite their availability, orthotopic models of human lung cancer are not widely used, and most of the research and development of novel therapeutics for lung cancer still relies upon s.c. tumor models, which are potentially less clinically relevant.

In this article, we describe the development of orthotopic models for different primary human lung cancers in athymic nude mice. We have developed models of each of the most common lung cancer histological types including adenocarcinoma, squamous cell, bronchioloalveolar, and small cell. For each tumor type, lesions develop after direct injection of a tumor cell suspension into the thorax of the mouse, making it a reproducible technique to study either NSCLC or SCLC human tumors. The present model recapitulates the local and regional growth pattern seen in lung cancer patients, *i.e.*, from a solitary

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[4] The abbreviations used are: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; i.t., intrathoracic; FBS, fetal bovine serum; GFP, green fluorescent protein; IHC, immunohistochemistry; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; IL, interleukin; VPF, vascular permeability factor.

nodule to a diffuse thoracic disease involving both lungs and the lymph nodes. Furthermore, in contrast to tumors growing s.c., tumors in the lung are less susceptible to treatment with paclitaxel, suggesting that orthotopic models are more relevant to evaluate chemotherapeutics and other therapies for human lung cancer.

MATERIALS AND METHODS

Cell Lines and Tissue Culture Conditions. Six human lung cancer cell lines were studied. NCI-H358 (bronchioloalveolar carcinoma), NCI-H1299 and A549 (poorly differentiated NSCLC), and NCI-H69 (SCLC) were obtained from the American Type Culture Collection (Manassas, VA). PC14PE6 was selected from human adenocarcinoma cell line PC14 to produce pleural effusion when injected into mice (18, 19). NCI-H226 (lung squamous cell carcinoma) was the gift of Dr. John D. Minna, University of Texas Southwestern Medical Center (Dallas, TX; Ref. 20). B16BL6 melanoma cells (21–23) were initially used to determine the feasibility of the orthotopic injection procedure. Floating aggregates of NCI-H69, floating and adherent monolayer cultures of PC14PE6, and adherent monolayer cultures of other cell lines were incubated at 37°C in 5% CO_2-95% air. NCI-H358 was cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin. NCI-H69 cells were cultured in Dulbecco's modified MEM supplemented with 10% FCS and penicillin-streptomycin. All of the other cell lines were cultured in Eagle's MEM supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, and penicillin-streptomycin mixture (CMEM; Flow Laboratories, Rockville, MD). All of the tumor cell cultures were free of *Mycoplasma*, and the following pathogenic murine viruses: retrovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Bethesda, MD).

Animals and Animal Care. Male athymic nude mice (NCr-nu) and C57BL/6 mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 6–10 weeks old.

Matrigel and Preparation of Cell Suspension for i.t. Injection. Matrigel is a basement membrane matrix preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma (Becton Dickinson & Co., San Jose, CA; Refs. 14, 16, 17). For all of the experiments a stock solution of 500 μg Matrigel in 1 ml PBS using a dilution factor of approximately ×30 according to compound concentration was used. Cell suspensions for thoracic injections were prepared of equal volumes of cells in PBS and Matrigel stock, giving final dilution factor of approximately ×60. Matrigel was thawed on ice to avoid gel formation, which can rapidly occur at room temperature or above. In accordance with the manufacturer's instructions, all of the cell line suspensions, syringes, and needles were kept on ice before injections. To prepare suspensions of tumor cells in Matrigel, adherent tumor cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% serum, and the cells were washed once in serum-free medium and resuspended in PBS. Floating cells were collected after centrifugation. Trypan blue staining was used to assess cell viability, and only single-cell suspensions of >90% viability were used for the injections. Both Matrigel matrix and growth factor-reduced Matrigel matrix were used.

i.t. Injection. Mice anesthetized with sodium pentobarbital (50 mg/kg body weight) were placed in the right lateral decubitus position. One-ml tuberculin syringes (Becton Dickinson) with 30-gauge hypodermic needles were used to inject the cell inoculum percutaneously into the left lateral thorax, at the lateral dorsal axillary line, ~1.5 cm above the lower rib line just below the inferior border of the scapula. The needle was quickly advanced 5–7 mm into the thorax and was quickly removed after the injection of cell suspension. After tumor injection, the mouse was turned to the left lateral decubitus position. Animals were observed for 45–60 min until fully recovered.

s.c. Flank Injection. For s.c. flank injections, unanesthetized mice underwent s.c. injection of cells suspended in a volume of 100 μl HBSS (Sigma Chemicals Co., St. Louis, MO) directly into the flank using 1-ml tuberculin syringes (Becton Dickinson) with 30-gauge hypodermic needles. For chemotherapy experiments, mice were injected with tumor cells in 150 μl PBS with Matrigel. The cell suspension was prepared as described above for i.t. injection. Mice were then examined daily for evidence of tumor development.

***In Vivo* Selection of Cell Lines for Increased Tumorigenicity.** Using the i.t. injection technique described above, NCI-H226 cells were injected into the lungs of nude mice. The mice were killed when moribund, and the largest thoracic tumors were harvested by aseptic techniques, dissociated mechanically using pipetting, and placed into culture for three to five passages (24). The cells were then reinjected into the lungs of nude mice.

***In Vitro* Selection for Increased Tumorigenicity: Growth in Semisolid Agarose.** NCI-H69 *in vitro* selection for increased invasive properties *in vivo* was accomplished using agarose, as described previously (25, 26). Briefly, agarose (Sigma Chemical Co.) was dissolved in distilled water and autoclaved. Base layers of Eagle's MEM with tryptose phosphate broth, 10% FBS, and 0.6% agarose were set in six-well plastic dishes. Over this bottom layer, a second layer of medium containing agarose and a suspension of 1×10^6 single tumor cells was laid. The concentration of agarose in the top layer was 0.9%. After the top layer gelled, 1–2 ml of CMEM medium with 10% FBS was added. Colonies formed from single cells were harvested and expanded by growth as monolayer cultures for *in vivo* injection.

GFP Transfection Protocol. For GFP transfection, cultures of PC14PE6 and NCI-H358 at 70% confluency were transfected with PEGFPC1 plasmid (Clontech Laboratories Inc.,

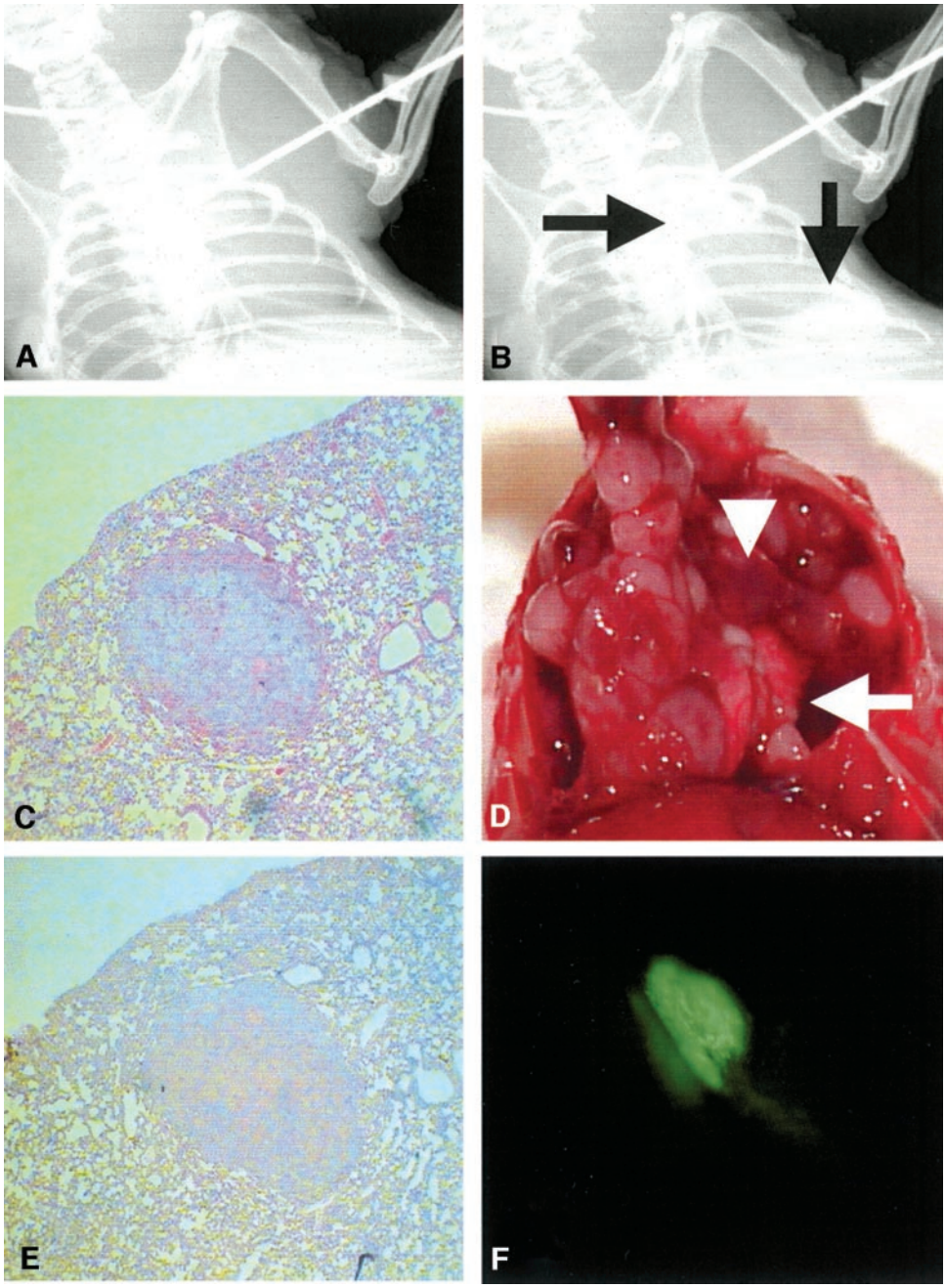

Fig. 1 *A*, to demonstrate fluid spreading after injection into the thorax, a 27.5-gauge needle was inserted into the left lung of a nude mouse (Faxitron X-ray image). *B*, the image was taken immediately after an injection of 75 μl of iohexol (omnipaque), an iodinated contrast agent. The fluid blunted the tip of the needle in the lung parenchyma and accumulated in the pleura according to gravity forces (*arrows*). *C*, the solitary nodules surrounded by a normal lung developed several days after injection of tumor cells with Matrigel, which anchored them and prevented cell suspension spread. PC14PE6 (*adenocarcinoma*) tumor, 9 days after tumor implantation. *D*, diffuse thoracic disease involving the injected site, the contralateral lung, and lymph nodes. Note the heart (arrowhead) and the small portion of the lung (*arrow*). *E*, IHC staining revealed that lung cancer in this system expressed bFGF from an early stage of disease. *F*, to study the sequence of metastasis we transfected two cell lines with GFP. The lesion is a microscopic left lung tumor on day 4 after injection of PC14PE6 cells with Matrigel. At this time, we found metastasis in the regional lymph nodes and the right lung.

Palo Alto, CA) using FuGene VI transfection reagents (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. After 48 h, the cells were harvested by a 0.25% trypsin-0.02% EDTA solution and placed at a ratio of 1:15 into selective medium containing 800 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) and plated. Neomycin-resistant clones were isolated with cloning cylinders by trypsin-EDTA. For *in vivo* studies, clones with high-intensity GFP fluorescence and stability were pooled. PC14PE6 cells (0.5×10^6) or NCI-H358 cells (1×10^6) in Matrigel were injected into the lungs of 10 mice. The mice (2/group) were killed at sequential time points thereafter.

Chemotherapy Studies. Therapeutic effects of paclitaxel were determined using NCI-H358 tumors implanted i.t. (1×10^6 cells in 75 μl) or s.c. (2.5×10^6 cells in 150 μl), or for PC14PE6 i.t. tumors (0.5×10^6 cells in 75 μl) or s.c. (1.5×10^6 cells in 150 μl). All of the cell suspensions were prepared in Matrigel. Four experiments were carried out with NCI-H358 i.t. tumors: one with s.c. tumors, one with PC14PE6 i.t, and two s.c. In all of the experiments, mice were randomized on day 7

Table 1 Production of thoracic tumors

Titration of human NSCLC and SCLC cell lines was conducted to determine the best number of cells needed to repeatedly produce orthotopic thoracic tumors in nude mice. Cells were injected in Matrigel into the left lung, as described in the text. Tumors metastasized to thoracic lymph nodes and the contralateral lung.

Cell line	Histology	Cell number	Tumor development	Survival (days)	Rt. lung metastases/ Lymph node metastases
PC14PE6	Adenocarcinoma[a]	0.5×10^6	10/10	26–30	+/+
		1×10^6	10/10	18–23	+/+
		2×10^6	10/10	14–18	+/+
NCI-H358	Bronchioloalveolar carcinoma	0.5×10^6	8/10	70–80	+/+
		1×10^6	9/10	56–63	+/+
		2×10^6	10/10	42–49	+/+
NCI-H226	Squamous	1.5×10^{6b}	10/10	63–70	+/+
		2×10^6	6/10	3.5–4 mo	+/+
NCI-H1299	Poorly diff NSCLC	1×10^6	10/10	32–40	+/+
A549	Poorly diff NSCLC	1×10^6	10/10	50–60	+/+
NCI-H69	SCLC	1.5×10^{6c}	9/10	50–56	+/+
		2×10^6	1/20	up to 4 mo	−/−

[a] PC14PE6 tumors were associated with pleural effusion. The amount of effusion collected was inversely related to number of cells injected as described in "Results."

[b] NCI-H226 cells were selected three cycles *in vivo* in the lung to improve tumor uptake and shorten survival time.

[c] NCI-H69 cells were selected on agarose for a more tumorigenic clone.

after tumor implantation to a control arm (i.p. 200 μl PBS/single dose/week) or a treatment arm [i.p. 200 μl paclitaxel, dosages ranged from 100–200 μg (4–8 mg/kg)/single dose/week]. For each of the cell lines, the experiment was terminated when i.t. control mice became moribund. Thus, all of the i.t. and s.c. mice for a particular cell line had the same number of chemotherapy cycles and, simultaneously, they were killed, autopsied, and tumor tissues were harvested.

Necropsy, Tissue Preparation, and IHC Staining. Mice were killed with a lethal dose of sodium pentobarbital (100 mg/kg body weight). Subsequent to a laparotomy, the thoracic cavity was inspected through the diaphragm for evidence of pleural effusion. Any pleural effusion was collected, and the thoracic organs were then removed enblock, including all of the lymph nodes and tumors. After dissection and removal of the heart, the lung and tumor mass were washed in cold PBS and weighed. Other visceral organs were removed and inspected for presence of metastases. Subcutis tumors were removed, washed in PBS, and weighed. For IHC and H&E staining procedures, one part of the tumor was fixed in formalin and embedded in paraffin, and another part was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored in −80°C. IHC determination of bFGF, VEGF/VPF, and IL-8 were performed as described previously (27).

Microscopy and Imaging. For studies of tumor cells transfected with GFP, 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). Digitalized imaging was performed using Faxitron specimen radiography system model MX-20 (Faxitron X-Ray Corp., Wheeling, IL). Energy was set to 26 kV, time to 10 s.

RESULTS

Formation of Lung Tumors. In the initial set of experiments, we determined the volume of tumor cell inoculum necessary to produce lung lesions without leading to immediate toxicity. For this purpose we used the highly metastatic B16BL6 melanoma cells, which were implanted into the lungs of syngeneic C57BL/6 mice. We selected the injection volume of 75 μl Matrigel containing suspended tumor cells. The necessity of Matrigel as an anchor to tumor cells, to avoid diffuse spread in the thorax, is demonstrated in Fig. 1, *A* and *B*. Injection of tumor cells in saline resulted in spread according to gravity forces, whereas injection of tumor cells with Matrigel formed a solitary lesion as an initial focus of disease. Four NSCLC cell lines (PC14PE6, NCI-H1299, NCI-H358, and A549) suspended in Matrigel were injected into the left lung and produced solitary lesions that progressed to diffuse thoracic disease.

The characteristics of tumor development and metastasis for the various human lung cancer cell lines are summarized in Table 1. The adenocarcinoma (PC14PE6) was the most rapidly growing tumor. Typically, 9 days after injection, solitary lesions could be detected in sections of lung (Fig. 1*C*). Diffuse thoracic growth (Fig. 1*D*) lead to death by 2.5–4.5 weeks after injection, depending on the number of cells injected. At the time of death, lymph node metastasis (bilateral axilla and neck) was evident. An inverse ratio was found between the number of tumor cells injected and production of pleural effusion. The injection of 0.5×10^6 cells yielded 8 lung nodules sized 1–2 mm with 0.8 ml bloody pleural effusion. The injection of 1×10^6 cells produced larger tumors that occupied 80% of the thorax with 0.2 ml bloody pleural effusion, and the injection of 2×10^6 cells produced rapid death from extensive tumor with no pleural effusion. In all of the cases, the formation of pleural effusion was associated with pleural seeding by tumor cells. NCI-H12999 (poorly differentiated NSCLC) also produced rapidly growing tumors, and the mice died with diffuse disease 5 weeks

Table 2 Paclitaxel therapy of bronchioloalveolar (NCI-H358) and adenocarcinoma (PC14PE6) i.t. and s.c. tumors

Cells were implanted orthotopically in the lungs or subcutis as described in the text. In all experiments, paclitaxel therapy began on day 7 after tumor implantation. For each of the cell lines, the experiment was terminated when i.t. control mice became moribund. More pronounced effect was noticed in the subcutis tumor in comparison to the thoracic tumors, suggesting that paclitaxel had limited effect on the thoracic tumors in this system.

	Control			Paclitaxel		
	Lung and tumor weight (g)			Lung and tumor weight (g)		
	Incidence[a]	Median	Range	Incidence	Median	Range
Bronchioalveolar thoracic tumors						
Study 1	10/10	0.307	0.219–0.515	10/10	0.266	0.192–0.342
Study 2	9/9	0.319	0.256–1.240	10/10	0.271	0.229–0.305
Study 3	9/9	0.345	0.266–1.313	9/9	0.215	0.171–0.338
Study 4[b]	9/9	0.340	0.190–0.399	8/9	0.248	0.180–0.329
Bronchioalveolar subcutis tumors						
Study 1[c]	5/5	0.412	0.225–0.662	5/5	0.085	0.025–0.220
Adenocarcinoma thoracic tumors						
Study 1	5/5	0.320	0.298–0.350	5/5	0.231	0.192–0.300
Adenocarcinoma subcutis tumors						
Study 1[c]	5/5	0.550	0.380–0.850	5/5	0.098	0.015–0.320
Study 2[c,d]	5/5	1.728	0.950–1.950	5/5	0.542	0.132–1.700

[a] Number of positive mice/number of mice survived the experiment.
[b] Paclitaxel dosage was 200 μg/mouse/week in all experiments except study 4 of NCI-H358 thoracic tumors in which it was 100 μg/mouse week.
[c] Tumor weight only reported.
[d] In the PC14PE6 subcutis experiments, 1.5×10^6 cells were injected in the first study and 2×10^6 cells in the second study.

after injection. NCI-H358 (bronchioloalveolar carcinoma) cells produced tumors with an intermediate growth rate as compared with the other cell lines that were studied. Solitary nodules were found 2 weeks after injection of 1×10^6 cells, and mice died of progressive disease 8–9 weeks later. A549 (poorly differentiated NSCLC) cells formed slow-growing tumors leading to cachexia characterized by wasting of the interscapular muscles. None of the mice studied became cyanotic, and mice were killed on the development of labored mouth breathing.

Tumorigenicity in Orthotopic and Ectopic Organs. Two human NSCLC cell lines suspended in saline or Matrigel were injected s.c. into the right flank of 5 mice/group. The rate of tumor development was similar in all of the mice. NCI-H358 cells developed s.c. tumors in 80% of mice injected with 2.5×10^6 cells, whereas 100% of mice injected i.t. with 2×10^6 cells developed lung tumors. PC14PE6 cells produced s.c. tumors in 80% of mice injected with 1×10^6 cells, whereas all of the mice injected i.t. with 0.5×10^6 cells developed lung tumors. Thoracic tumors were fatal in all of the mice, whereas s.c. tumors were not associated with mortality when tumors reached maximal permissible size.

Serial *in Vivo* Passages for Selection of Cells with Increased Tumorigenicity. The squamous cell carcinoma cell line NCI-H226 produced slow-growing tumors with no pleural effusion. Injection of 0.5×10^6 or 1×10^6 cells did not form visible tumors up to 4 months after tumor implantation. Six of 10 mice injected i.t. with 2×10^6 cells developed a thoracic tumor and became moribund 3.5–4 months after tumor injection. The largest tumors were harvested and established *in vitro*. Viable cells were harvested and reinjected into the lungs of nude mice. After three such selection cycles, a cell line with an increased tumorigenicity was isolated (Table 1).

***In Vitro* Agarose Selection Enhanced the *in Vivo* Tumorigenicity of NCI-H69 Cells.** The injection of human SCLC NCI-H69 (0.5×10^6, 1×10^6, and 2×10^6 cells) into the lungs of nude mice did not form tumors. In 1 mouse injected i.t. with 2×10^6 cells, a 2-mm tumor was found 12 weeks after injection. To enhance the invasive potential of the parental NCI-H69 cell line, a method of *in vitro* selection using anchorage-independent growth of cells in agarose (25, 26) was used. The selection process was twice repeated using higher concentrations of top layer agarose from 0.9% to 1.2% (Table 1). After two cycles of selection in agarose, tumor cells formed lung tumors in all of the mice injected i.t.

Lung and Lymphatic Metastasis of NSCLC Tumor Cells After i.t. Injection. To study tumor progression and metastasis, adenocarcinoma (PC14PE6) and bronchioloalveolar carcinoma (NCI-H358) cells were transfected with GFP. Ten mice were injected with either 0.5×10^6 or 1×10^6 cells in Matrigel, into the left lung as described above. Two mice were killed at 4–7-day intervals. All of the mice injected developed microscopic tumors identified by fluorescence microscopy (Fig. 1*F*). PC14PE6 progressed to mediastinal lymph nodes and spread to the right lung on day 4 after tumor implantation. In NCI-H358 tumors, lymph node and right lung lesions were detected on day 28 after injection.

Chemotherapy Study. We studied the effect of paclitaxel on NCI-H358 (bronchioloalveolar carcinoma) and PC14PE6 (adenocarcinoma) tumors, growing in the lungs or the subcutis. In all of the experiments, therapy began on day 7 after tumor inoculation. We conducted several experiments using 100 or 200 μg (4 or 8 mg/kg)/dose paclitaxel. Therapy was administered for up to five cycles or until control mice became moribund. Mice injected with NCI-H358 were treated for five cycles and killed 7.5–9 weeks after tumor injection. Mice injected with PC14PE6 cells, a more aggres-

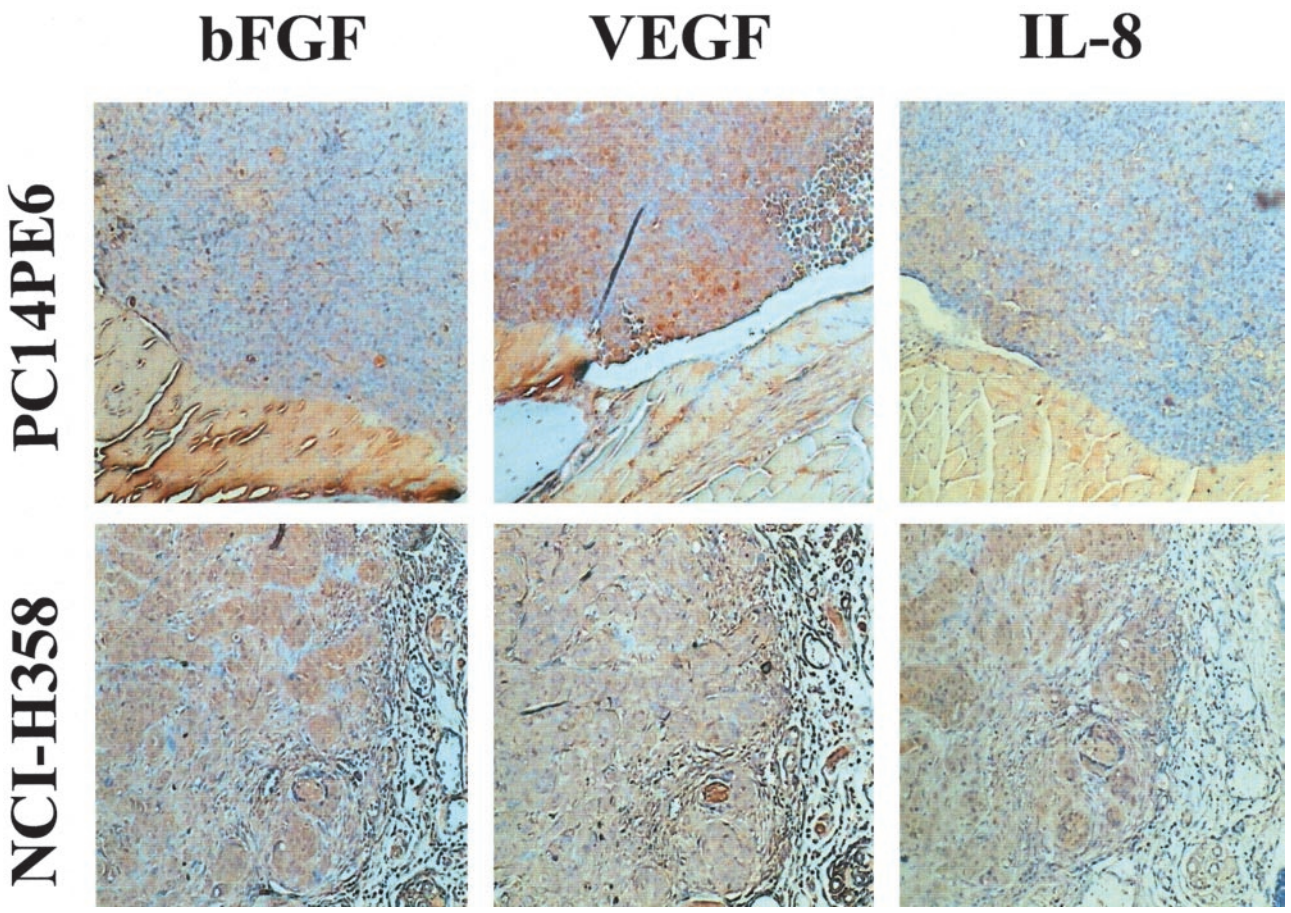

Fig. 2 IHC staining for bFGF, VEGF/VPF, and IL-8 of PC14PE6 and NCI-H358 orthotopic tumors. Although all of the factors were expressed by the two tumors, VEGF/VPF expression was prominent in PC14PE6 tumor, an adenocarcinoma that produces pleural effusion.

sive cell line, were treated for four cycles and were killed 4 weeks after tumor implantation. Control mice in the s.c. injected groups did not have evidence of morbidity at the end of the experiment. All of the mice in all of the groups tolerated therapy well, as assessed by similar median body weights in control *versus* treatment groups (data not shown). The results of all of these experiments suggest that whereas paclitaxel is efficacious in controlling tumor growth in the lungs, it is much more effective for treating s.c. tumors (Table 2). The treatment results of the thoracic tumors did not vary significantly when using 100 or 200 μg/dose paclitaxel. Similarly, the drug effect did not change significantly when by 7.5 or 9 weeks after tumor implantation.

Immunohistochemical Analysis of Thoracic Tumors. Thoracic tumors were examined for expression of several proangiogenic factors including bFGF, IL-8, and VEGF/VPF. These factors were expressed at an early stage of tumor progression, as noted by positive staining for bFGF in PC14PE6 tumors on day 9 after injection (Fig. 1*E*). A differential pattern of expression for each growth factor was noted between the different types of lung cancer. The pleural effusion producing adenocarcinoma (PC14PE6) tumors expressed higher levels of VEGF/VPF as compared with IL-8 and bFGF. These three factors were also expressed by the bronchioloalveolar tumors (NCI-H358), although to similar degree (Fig. 2). The NCI-H358 tumor had low expression of E-cadherin and high expression of matrix metalloproteinase 2 (data not shown).

DISCUSSION

We have developed reliable and reproducible orthotopic nude mouse models of lung cancer in a stepwise fashion. First, we verified the feasibility of thoracic injection and the development of regional metastasis using the pigmented B16BL6 melanoma cell line in syngeneic mice. Next, four different human lung tumor lines were injected into the lung of nude mice: PC14PE6, a pleural effusion producing adenocarcinoma, NCI-H358 bronchioloalveolar carcinoma, and NCI-H1299 and A549 poorly differentiated NSCLC lines. Two additional tumor lines developed tumors after selection for more aggressive and invasive clones: NCI-H226 squamous cell carcinoma was selected *in vivo* in the lung using our model, and NCI-H69 small cell lung carcinoma was selected on agarose *in vitro*. For each of the lung cancer cell lines studied, tumors formed as a single focus at the site of injection into the lung. Tumors then grew progressively within the injected lung and spread to regional and

extrathoracic lymph nodes, and the contralateral lung. The pattern of spread of lung cancer within the thorax is similar to that observed clinically in patients with lung cancer, suggesting that the model is clinically relevant. The absence of clinically apparent distant metastasis is most likely due to the aggressive pattern of locoregional spread of cancer cells with mice dying of diffuse thoracic disease before development of significant distant metastasis. To overcome this potential limitation, complementary models of metastatic disease (*i.e.*, brain, liver, and bone models) are now being developed for each of the lung cancer types.

This model validates the orthotopic principle that tumor cells grow better in their tissue of origin and that more clinically relevant studies can be performed using the orthotopic site of tumor growth. The importance of orthotopic models to study the biology and therapy of cancer had been demonstrated for other neoplasms (4, 7). Chen *et al.* (28) studied the interaction between NSCLC tumors and macrophages in surgical specimens and in cell lines, and found that the importance of tumor cell and microenvironment interaction holds also for the lung. They suggested that this interaction up-regulated the expression of IL-8 by the tumor. Furthermore, high-density infiltration of tumor by macrophages was correlated with increased tumor angiogenesis and adverse outcome in NSCLC patients. Moreover, our model closely resembles human lung cancer in its partial response to chemotherapy. Although treated mice had shown a reduction in tumor burden in response to paclitaxel (as assessed by lung and tumor weight), a more dramatic response was noted in s.c. tumors. This effect of paclitaxel on s.c. implanted tumors may in part explain the difficulty with the translation into a clinical reality of dramatic responses to therapy that have been observed for anticancer agents in preclinical studies that rely solely on s.c. tumor xenografts (6). Therefore, it may be prudent to first screen novel anticancer agents in s.c. tumor models and to then screen active agents in orthotopic models before the initiation of clinical trials.

To date, several orthotopic rodent models have been developed to study human lung cancer. Different techniques have been used to introduce tumors including intrabronchial implantation of a tumor cell inoculum (9–11) with resultant tumor formation in the center of the thorax in 35–95% of animals. However, intrabronchial techniques necessitated irradiation and tracheostomy or laryngoscopy and were associated with operative mortality of >5%. Alternatively, intrapleural implantation of a tumor cell inoculum without Matrigel has been used (12, 13), which resulted in the development of extrathoracic tumors and an operative mortality of 5–10%. Tumors can also be implanted in the lung parenchyma after skin incision (14) or thoracotomy and surgical exploration of the pleura (15–17). The skin incision model was associated with low operative mortality yet was much more time consuming and laborious, whereas the thoracotomy model was associated with mortality of about 5%. Another common technique used surgical human cancerous specimens, which were implanted into the lungs (8). However, in this system the number of tumor cells varies between surgical specimens. To date, none of these models had been widely accepted, and most research is still done using s.c. models.

The models of lung cancer that we describe complement those that have been developed previously and may overcome some of the limitations associated with them. We have developed orthotopic models of each of the common types of lung cancer, which should lead to an improved understanding of the influence of tumor histology on response to existing and emerging therapies. The techniques needed for our models are reproducible, can be performed quickly (*i.e.*, each mouse can be injected in under 15 s), and are easily taught. It is associated with virtually no procedure related animal mortality. Whereas pneumothorax may occur, as shown in 3 of 10 treated mice studied with X-ray imaging (Faxitron; data not shown), death within 72 h of the thorax puncture is <1%. Matrigel is used to provide a reproducible anchor, which fixes the tumor cells to the site of injection and avoid cell dispersion. In most experiments we used growth factor-reduced Matrigel. It contains limited amounts of growth factors, which are additionally diluted in the process of cell suspension preparation. Overall tumor cell implantation with its addition results in a reproducible tumor size, which enables therapeutic experiments as demonstrated with paclitaxel as well as study of tumor biology and metastasis.

Of special interest is the expression of proangiogenic factors by the implanted lung tumors. The significance of bFGF, VEGF/VPF, and IL-8 in human NSCLC had been studied extensively, and their expression had been shown to correlate with poor outcome in human lung cancer patients (29). These factors are important for tumor progression and the formation of the angiogenic phenotype from an early stage disease. In our model of lung adenocarcinoma, bFGF was expressed by early and locally advanced human lesions in the lungs of nude mice. These data are consistent with what has been observed in the clinic, because bFGF was shown to play a key role in human lung cancer progression (30) and was expressed even in small tumors (≤2 cm) growth (31). Taken together, the data suggest that it may be prudent to target bFGF for lung adenocarcinoma using anti-bFGF therapies such as low dose-daily IFN (32). In our model of bronchioloalveolar carcinoma (NCI-H358), tumors were found to have low expression of E-cadherin (associated with cell-to-cell cohesion and adhesion) and high expression of matrix metalloproteinase 2 (associated with invasion). This proportion had been found to correlate with increased human lung cancer tumor aggressiveness (33), which substantiates the metastatic behavior of this tumor. Interestingly, VEGF/VPF expression was higher in lung adenocarcinomas (PC14PE6) than for other lung cancer histologies, which may be related to production of pleural effusion by PC14PE6 tumors. Indeed, Senger *et al.* (34) had identified this molecule by its ability to induce vascular leaking and named it vascular permeability factor. Yano *et al.* (19) had found the relation between VEGF/VPF and PC14PE6 tumors in a metastatic tumor and, as far as we know, our finding is the first in an orthotopic model.

In summary, we have developed *in vivo* models of primary lung cancer for both human NSCLC and human SCLC, which are reproducible, well tolerated, feasible, and straightforward to perform. These lung cancer models closely mimic the patterns observed for the natural progression of primary lung cancer from a single nodule to disseminated disease, and enables study of novel therapeutics and better understanding of lung cancer metastasis.

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