Irradiated Nude Rat Model for Orthotopic Human Lung Cancers

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ABSTRACT

The development of improved animal models for biological and preclinical studies of human lung cancer is important because lung cancer is the leading cause of cancer death in the United States. To determine whether the Rowett nude rat could serve as an orthotopic (organ-specific) model of this disease, nude rats (CR: NIH-RNU), with and without 500 rads of prior γ-irradiation, were implanted intrabronchially with 10^7 cultured cells from 3 human lung cancer lines. Without irradiation, the NCI-H460 large-cell undifferentiated carcinoma had a 54% take-rate, whereas the NCI-H125 adenosquamous carcinoma and A549 adenocarcinoma had take-rates of 7 and 33%, respectively; irradiation increased the respective take-rates to 100, 83, and 90%. In irradiated rats, tumor age versus weight measurements showed progressive growth for all three tumors, with growth rates in the order: NCI-H460 > A549 > NCI-H125, requiring approximately 3, 5, and 9 weeks, respectively, for average tumor sizes to exceed 500 mg. The small-cell carcinoma cell line NCI-H345 was implanted only into irradiated rats and resulted in more slowly growing tumors. Histopathological study showed all model tumor types to have histological characteristics consistent with the clinical tumors from which the cell lines were derived. Each tumor type had a different growth pattern, with some of the the A549- and NCI-H125-derived tumors metastasizing to contralateral lung and/or regional lymph nodes. There was no evidence for immunological rejection in irradiated, tumor-bearing rats. Nonirradiated, implanted rats without gross tumor exhibited peribronchiolar mononuclear cell infiltration with or without fibrosis, suggesting prior immunological rejection. The successful orthotopic growth of these 4 human lung cancer cell lines in irradiated nude rats suggests that this model could be useful for biological and preclinical studies of human lung cancer, both in intact rats and via ex vivo perfusion of their tumor-bearing lungs.

INTRODUCTION

A rising incidence combined with a lack of effective methods for either early detection or treatment has made lung cancer the leading cause of cancer death in the United States (1–4). It is therefore imperative to develop improved animal models of this disease for in vivo biological and preclinical studies. Both s.c. (5–7) and subrenal capsule (8–10) rodent models have previously been used for such studies, but it is now appreciated that nude rodents with human tumor material implanted in orthotopic (organ-specific) sites offer better tumorigenicity and metastatic potential than these ectopic (abnormally positioned) models (5, 11–19). In addition to the improved modeling of human cancer biology, orthotopic studies might also better model the pharmacokinetic compartments and pharmacodynamics relevant to treatment of human cancers (11).

Orthotopic nude mouse models have recently been developed for a number of human cancers, including those of the lung, colon, pancreas, kidney, brain, and skin (11–19). The orthotopic mouse model for lung cancer developed by McLemore et al. (11, 12) has thus far been used primarily for comparative modeling studies. Using this model, it has been shown that (a) a variety of cultured human cancer cell lines, as well as some enzymatically disaggregated clinical specimens, can be successfully propagated by i.b. implantation; (b) human lung cancer cell lines implanted i.b. frequently exhibit mediastinal invasion; (c) i.b. cell implantation requires fewer cells and results in much higher tumor-related mortality than does s.c. implantation; and (d) the histological characteristics of i.b. implanted cell lines resemble those of the parent tumor from which the cell lines were derived. The efficient propagation, mediastinal invasion, and lethality seen in this orthotopic mouse model suggest that it should provide clear advantages over previously used models for in vivo biological and preclinical study of human lung cancer.

On the other hand, the nude mouse model is not ideal for some applications because of its small size; the nude rat (20) is often more convenient. For in vivo experimentation, nude rats (a) more readily allow surgical procedures and/or repeated blood sampling and (b) can carry a much greater tumor burden (particularly with orthotopic tumors), thereby increasing both the time available to study the tumor and the amount of tumor tissue obtainable. In addition, the considerably larger lung size of nude rats facilitates ex vivo perfusion of their tumor-bearing lungs, a technique for performing well-controlled biological and preclinical studies of in situ orthotopic human lung cancers.4

A disadvantage of Rowett nude rats is their relative immunocompetence compared to nude mice. Previous studies of nude rats given ectopic injections (s.c.) of human tumor material have shown reduced take rates compared to nude mice and a tendency for the tumors to spontaneously regress (20–26). It is unknown, however, whether nude rats can be used for orthotopic (i.b.) human lung cancer growth and whether additional immunosuppression would be beneficial in this rat model. We have modified the i.b. implantation method described by McLemore et al. (11, 12) to answer these questions.

MATERIALS AND METHODS

Human Tumor Cell Lines. Human lung cancer cell lines NCI-H125, NCI-H460, NCI-H345, and A549 (27) were obtained from Drs. J. Minna, A. Gazdar, and J. Mayo (National Cancer Institute, Frederick Cancer Research Facility). All cell lines were recovered from cryopreserved seed stock and cultivated in standard tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA) without antibiotics. Cells were maintained at 37°C in a humidified incubator gassed with 5% CO2 in air. When cells growing in monolayers were 60–80% confluent, they were subcultured or harvested for implantation using trypsin–EDTA (Sigma, St. Louis, MO).

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3The abbreviation used is: i.b., intrabronchial.

Cells to be implanted were washed twice in RPMI 1640, counted with a hemocytometer, and adjusted to the correct concentration of trypan blue-viable cells in 100 μl of the same medium. All cell lines were previously shown to be human by karyotype analysis and were regularly screened for Mycoplasma infection.

Animals. Male or female nude (CR: NIH-RNU) rats (obtained from the National Cancer Institute, Frederick Cancer Research Facility) were received in the nude rodent reverse isolation facility at the University of Colorado Health Sciences Center at 4 weeks of age and acclimated for 1 week before entering study protocols. Rats were kept in previously sterilized filter-topped cages and fed autoclaved food and water ad libitum. Manipulations were done under sterile conditions in a laminar flow hood. All studies had previously been approved by the institutional Animal Care and Use Committee.

Tumor Cell Implantation. On the morning of the day of implantation, 5-week-old rats to be irradiated were given 500 rads of whole-body γ-irradiation from a 60Co source (Atomic Energy of Canada Limited γ-Beam 150 Irradiator; Ontario, Canada) at 150 rads/min while confined upright in a gas-sterilized plastic holding apparatus (Harvard Bioscience, South Natick, MA). That afternoon, all rats were anesthetized i.m. with ketamine/xylazine (Parke-Davis, Morris Plains, NJ; Mobay, Shawnee, KS; 80 and 12 mg/kg, respectively) and intratracheally implanted (11, 12) with 10⁶ tumor cells using a 20-gauge, 2-inch-long Teflon catheter (Deseret Medical, Inc., Sandy, UT) passed into the right caudal lobe via a small tracheostomy incision. After closing the wound, sterile clips and recovery in cages warmed on heating pads, the rats were returned to their shelves and treated prophylactically with Augmentin (Beecham Labs, Bristol, TN) at 0.35 mg/ml drinking water for 2 weeks.

Determination of Take-rates. Take-rates were measured in irradiated and nonirradiated rats by determining what fraction of rats implanted with each cell line had evidence of gross tumor after a period previously shown to allow the development of sizable (>0.5 g) tumors in irradiated rats. The times required for tumor development were: NCI-H460, 3 weeks; A549, 5 weeks; NCI-H125, 10 weeks.

The protocol was as follows. On at least three separate occasions, groups of rats (3–8 each) were implanted with each cell line, with and without prior irradiation. The animals were visually monitored 3 times/week for evidence of tumor development. Rats exhibiting early morbidity (5–10%) were euthanized by an overdose of ketamine/xylazine and autopsied; those dying unexpectedly before their scheduled sacrifice (5–10%) were also autopsied. Autopsied rats in these categories with obvious tumors were included in the study. Such animals without obvious tumors were excluded since it was impossible to determine whether the tumor would have developed if the rat had survived until the originally intended time of sacrifice. About 5% of the rats died from tumors growing in the pleural space; they were considered failed implantations and excluded from the study. To determine whether there was tumor development subsequently followed by regression, each animal was radiographed (see below) every 2–3 weeks until sacrifice.

At the predetermined intervals, all animals were euthanized as above and their heart-lung blocks were removed. Each lung lobe was palpated for evidence of a tumor. In cases where tumors were clearly present they were either removed and weighed or fixed in the intact heart-lung block for histopathological characterization (see below). When tumors were not clearly determined to be present by palpation, most heart-lung blocks were separated into individual lung lobes which were then individually cut into 1–2-mm-wide strips for more careful examination. However, at least 2 nonirradiated rats, implanted with each cell line and without palpable tumors, were fixed for histopathological characterization of any inflammatory or immunological reaction at the implantation site. Since nonirradiated NCI-H125-implanted rats require about 10 weeks for sizable tumor growth, it was uncertain whether any inflammatory or immunological response occurring immediately in these animals would still be visible at 10 weeks. Therefore, 2 of these animals were also euthanized early, at 2 weeks postimplantation, for histological characterization.

Determination of Growth Rates. Groups of irradiated rats (3–8 each) were implanted with each cell line on at least 3 different occasions.

Animals were visually monitored 3 times/week; those exhibiting early morbidity (about 5%) were euthanized and excluded from the study; those dying suddenly (also about 5%) were likewise excluded. All other rats were randomly sacrificed and autopsied, and their tumors were weighed at different time intervals as follows: NCI-H460, 10–19 or 20–29 days; A549, 20–29, 30–39, or 40–49 days; NCI-H125, 40–49, 50–59, or 60–69 days. A graph of average tumor weight versus time interval was constructed.

Chest Roentgenographic Studies. Ketamine/xylazine-anesthetized rats were radiographed in anteroposterior and right lateral recumbent positions using a conventional radiographic unit (GX 1050; Picker International, Inc., Highland Heights, OH) with a 1.2-mm nominal focal spot and a retractable mirror. The receptor was a double emulsion mammographic type (T-Mat M film and Min-R fast screens; Eastman Kodak Co., Rochester, NY) used without a grid. Typical exposure factors were 29 kVp, 300 mA, and 17 msec, with the mirror retracted. The limit of detection was a tumor diameter of approximately 3 mm.

Histopathological Study. To characterize the growth patterns of each type of tumor, heart-lung blocks were removed from a number of euthanized animals with gross evidence of tumor; the lungs were fixed by tracheal perfusion with 20 ml of 10% buffered formalin and placed in a container of the same solution for at least 48 h. All lung lobes and mediastinum were embedded in paraffin, stained with H & E, and examined in a blinded fashion by a board-certified pathologist (H. C.). In addition, the lungs of at least 2 nonirradiated rats implanted with each cell line and found to be without tumors at such times as tumors were normally expected in irradiated rats were similarly fixed and stained, and the right caudal lobe was then examined for evidence of inflammatory or immunological reaction or microscopic tumor at the implantation site.

RESULTS

Take-rate estimations were based on the results of both radiography and gross autopsy follow-up of each animal. Each rat was radiographed³ (Fig. 1) periodically and then sacrificed and autopsied after an interval previously shown sufficient for that particular cell line to grow to a tumor size of approximately 500 mg. Every rat radiographically positive for tumor was later found to have gross tumor at autopsy; therefore the possibility that tumors grew and then regressed before sacrifice was excluded.

Table 1 summarizes the tumor take-rates for the three different human lung cancer cell lines implanted intrabronchially with and without prior irradiation. The NCI-H125 adenocarcinoma cell line had a take-rate of 7% in untreated rats and 83% with prior irradiation. Large-cell carcinomas arising from the NCI-H460 large-cell line were present in 54% of the rats without and 100% of the rats with prior irradiation. The third cell line, the A549 adenocarcinoma, had a 33% take-rate without pretreatment and improved to 90% with prior irradiation. Since completing this study, rats have routinely been irradiated prior to implantation with each of these cell lines; take-rates have consistently been comparable to those shown here.

Over 1000 rats have received injections thus far, with a surgical mortality rate of 5–10%. Approximately 90% of confirmed tumors were found in the right caudal lobe; most tumors arising outside the right caudal lobe were found in the pleural space. These “extrapulmonary” tumors were presumed to arise from an injection made after accidentally passing the cannula through the visceral pleura; such tumors with the NCI-H460 cell line tended to be particularly aggressive and rapidly killed the rats by lung compression. A very small percentage of tumors

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Fig. 1. Roentgenogram of a nude rat right caudal lobe lung tumor 6 mm in diameter arising from NCI-H460 cells. The tumor is clearly visible (arrows) in both anteroposterior (a) and right lateral (b) views.

Table 1 Intrabronchial tumor take-rates for nude rats with or without prior γ-irradiation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Take-rate (%)</th>
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<tbody>
<tr>
<td>NCI-H125</td>
<td>None</td>
<td>1/15 (7)</td>
</tr>
<tr>
<td></td>
<td>500γ</td>
<td>19/23 (83)</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>None</td>
<td>7/13 (54)</td>
</tr>
<tr>
<td></td>
<td>500γ</td>
<td>21/23 (100)</td>
</tr>
<tr>
<td>A549</td>
<td>None</td>
<td>6/18 (33)</td>
</tr>
<tr>
<td></td>
<td>500γ</td>
<td>19/21 (90)</td>
</tr>
</tbody>
</table>

* Determined by gross inspection of 1–2 mm thick lung slices at the following times postimplantation: NCI-H460, 3 weeks; A549, 5 weeks; NCI-H125, 10 weeks.


* Significantly different from untreated at P ≤ 0.05 (Fisher’s exact test).

(<3%) were found within other lung lobes, usually on the right side. Occasionally, small tumors were also found in the s.c. tissue surrounding the tracheostomy site.

Each of the three tumor types shown in Table 1 had different growth rates and distinctive cytological and histological characteristics. Tumors from the NCI-H125 adenosquamous cells grew the slowest (Fig. 2), requiring 9 or 10 weeks to produce a tumor weighing approximately 500 mg. Histologically (Fig. 3), within the right caudal lobe these tumors grew in both parenchyma and bronchi and formed nests with the appearance of poorly differentiated adenocarcinomas (n = 6); there was no evidence of squamous differentiation in any of the tumors examined. Three of four heart-lung blocks examined showed metastasis to the left lung, and 2 of 4 had mediastinal lymph node metastases. This was the only cell line of the four lung cancer cell lines examined where left lung metastases were observed. None of the tumors showed evidence of immunological rejection, and necrosis was minimal.

Tumors arising from the NCI-H460 large-cell line grew very rapidly (Fig. 2), requiring 2 to 3 weeks to reach the 500-mg size range. If not euthanized, most of these rats died from their tumors within 4 weeks. Right caudal lobe tumors from this cell

Fig. 2. Tumor weight versus age data for tumors arising from i.b. implantation of 10⁷ cells from three different cultured human lung cancer cell lines into irradiated rats: NCI-H460 large-cell carcinoma, A549 adenocarcinoma, and NCI-H125 adenosquamous carcinoma. The numbers above each column are the number of animals per group. Columns, mean; bars, SE.

Fig. 3. Light micrographs of a 9-week-old, H & E-stained, right caudal lobe tumor arising from i.b. implantation of NCI-H125 cells. a, tumor invading the parenchyma from a large airway. Cancer cell deposits represent either metastases or aerogenous spread (arrows). × 9. Similar deposits were also found in the left lung of rats with this tumor type. b, nest of cancer cells growing among other less organized cancer cells, typical of a poorly differentiated adenocarcinoma. × 350.
line manifested as large-cell undifferentiated carcinomas growing in both airway and parenchyma \((n = 6)\), but especially within the airway (Fig. 4). Necrosis was common and was estimated to range from 10 to 40%. No evidence of lung or lymph node metastasis or immunological rejection was seen in any of the rats.

The A549 adenocarcinoma cells gave rise to tumors growing at an intermediate rate (Fig. 2), producing a 500-mg tumor in 4 to 5 weeks. Right caudal lobe tumors from this cell line also appeared as poorly differentiated adenocarcinomas \((n = 5)\) growing in both the parenchyma and the airway (Fig. 5). Cellular heterogeneity was seen in tumors from this cell line; a light-colored foamy cell predominated, but clusters of darkly staining cells were also seen. Tumors ranged from less than 10% to over 40% necrotic but showed no evidence of immunological rejection. Lung metastases were not seen, but 2 of 5 animals had mediastinal lymph node metastases.

Cells from a human small-cell carcinoma, NCI-H345, were implanted only into irradiated rats. A total of 8 rats received implantations. No tumor was found at autopsy in single rats euthanized at 3 and 6 weeks postimplantation. In four rats euthanized at 8 weeks, one had a small (4-mm-diameter) tumor. Two remaining rats, which were radiographically negative at 7 weeks, became positive at 10 weeks. Autopsy of these rats at 11 weeks confirmed the presence of medium-sized (~300 mg) tumors in the right caudal lobes. Histological study (Fig. 6) showed growth in both parenchyma and bronchi. Both had typical small-cell cytology, little necrosis, no apparent tendency to metastasize, and no evidence of immunological rejection.

Microscopic examination of the right caudal lobes of 6 non-irradiated rats implanted with NCI-H125 cells, A549 cells, or NCI-H460 cells (2 rats with each) and without gross evidence of tumor at 10, 5, and 3 weeks postimplantation, respectively, revealed local inflammatory responses consistent with immunological rejection in all rats. Fig. 7a shows peribronchiolar mononuclear cell infiltration in a rat implanted 10 weeks earlier with NCI-H125 cells. Peribronchiolar fibrosis accompanied by mononuclear cell infiltration 5 weeks after implantation of A549 cells is seen in Fig. 7b.

Two weeks after implantation of NCI-H125 cells, 2 rats were sacrificed to look for early evidence of immunological rejection, in case the evidence was no longer present at 10 weeks in this more slowly growing tumor. Both rats showed peribronchiolar mononuclear cell invasion; one of them had a small in situ tumor, bordered by an area containing mononuclear cells and fibrosis (Fig. 7c).

**DISCUSSION**

In this study we have implanted cultured human cancer cell lines intrabronchially into Rowett nude rats to explore the utility of these rats for growing orthotopic human lung cancer xenografts. The effect of additional immunosuppression was examined for three cell lines: a large-cell carcinoma (NCI-H460) and two adenocarcinomas (NCI-H125 and A549). Prior treatment with 500 rads of \(\gamma\)-radiation resulted in take-rates...
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Fig. 6. Light micrographs of an 11-week-old, H & E-stained, right caudal lobe tumor arising from i.b. implantation of NCI-H345 cells. a. local parenchymal growth of a tumor with very little necrosis. × 9. b. small cancer cells with small nuclei and salt-and-pepper chromatin typical of a small-cell carcinoma. × 350.

above 82% for all three cell lines. Without irradiation, take-rates were below 34% for the two adenocarcinomas. All tumors examined had a cytology and histology consistent with those of the parent tumors from which the cell lines were derived. Each type of tumor showed distinct growth patterns and rates; none showed any evidence of immunological rejection. Some NCI-H125- and A549-derived tumors metastasized within the thorax. In nonirradiated rats in which gross evidence of tumor was lacking, peribronchial fibrosis and/or mononuclear cell infiltration were seen in the right caudal lobe.

The relative order of take-rates in nonirradiated rats (NCI-H460 > A549 > NCI-H125; Table 1) was the same as the relative order of growth rates in irradiated rats (Fig. 2). This suggests that the growth rate may be the major factor determining take-rates in nonirradiated Rowett nude rats, but cell line-specific factors such as antigenicity could also be important. Take-rates for s.c. injections of human tumor material are also dependent on the cell or tissue type implanted (20–24).

Evidence presented here that whole-body irradiation significantly increased i.b. lung tumor take-rates (Table 1) is also consistent with previous reports showing increased take-rates for s.c. tumor xenografts after irradiation in either nude rats or mice (26, 28). The mechanism by which irradiation facilitates ectopic and orthotopic tumor establishment in nude rodents is not precisely known. Since natural killer cells, macrophages, and plasma cells are relatively resistant to such treatment, it seems more likely that damage to some B- or “T-like” cell population might be relevant (for a discussion, see Ref. 29). Regardless of the mechanism involved, our high take-rates suggest that Rowett nude rats, irradiated 2 to 6 hours before cell implantation, are a practical animal model for the i.b. growth of a variety of human lung cancer cell lines.

Microscopic analysis of these orthotopic lung tumors showed both histological and cytological characteristics consistent with the tumor from which the cell line was originally derived (Figs. 3–6). Others have shown that tumors or cell lines implanted s.c. into nude rats also maintain an appearance similar to that of the parent tumor (20, 23, 25, 30–31), although stromal differences, especially tumor encapsulation, are frequently seen in s.c. but not orthotopic tumor xenografts (14–16).

Fig. 7. Light micrographs of H & E-stained, right caudal lung lobes of mutin,nu.uni rats showing evidence of mononuclear cell infiltration and/or fibrosis without gross tumor. a. peribronchiolar mononuclear cell infiltration (arrows) 10 weeks after NCI-H125 cell implantation. × 40. b. area adjacent to an airway 5 weeks after A549 cell implantation. × 40. Immediately below the airway epithelium is an area of fibrosis (F); nearby airway smooth muscle (arrows) with associated mononuclear cells. c. lung tissue taken 2 weeks after implantation of NCI-H125 cells reveals a small in situ tumor (arrows) next to normal airway epithelium; below the tumor and epithelium is an area of fibrosis and mononuclear cell infiltration. × 250.
Unlike ectopic tumors previously described in the s.c. nude rat model (21, 25, 26, 30), two of our orthotopic tumor types (NCI-H125 and A549) exhibited metastasis. Both cell lines gave rise to regional lymph node metastases in about half of the tumor cells in the implantation syringe, or accidental growth requirements of these cell lines, incomplete expulsion of mononuclear cells and fibrosis directly associated with the rejection is the primary reason for lack of tumor take in non-vascularization in the rapidly growing tumors, but it could also be due to interspecies variation.

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