Comparison of Intrapulmonary, Percutaneous Intrathoracic, and Subcutaneous Models for the Propagation of Human Pulmonary and Nonpulmonary Cancer Cell Lines in Athymic Nude Mice


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ABSTRACT

The propagation efficiencies, growth patterns, histological appearances, and roentgenographic demonstration of tumors derived from six continuous human pulmonary tumor cell lines implanted intrathoracically (i.t.) and intrabronchially (i.b.) were compared with the conventional s.c. implantation method at three different tumor cell inocula (N = 184, i.b.; N = 185, i.t.; N = 180, s.c.). A tumor-related mortality of 100% was noted when the six different human lung tumor cell lines, including A549 adenocarcinoma, NCI-H125 adenocarcinoma, NCI-H1660 large cell undifferentiated carcinoma, NCI-H157 small cell carcinoma, and NCI-H358 and NCI-H322 bronchioloalveolar cell carcinomas, were implanted i.t. at a 1.0 x 10⁶ tumor cell inoculum. A similar (92%) tumor-related mortality was observed when these same lung tumor cell lines were implanted i.t. at a 1.0 x 10⁵ tumor cell inoculum (P > 0.10), whereas minimal (5%) tumor-related mortality was noted when cells from the six different cell lines were implanted s.c. (P < 0.001). In addition, a dose-dependent, tumor-related mortality was noted for either i.t. or i.b. implantation when lower (1.0 x 10⁴ or 1.0 x 10⁵) tumor cell inocula were employed. Histological characteristics and growth patterns of tumors propagated employing the three implantation techniques were closely comparable for all three propagation methods and, in all instances, histological appearances of the tumors were representative of the current tumor cell lines from which they were derived. Approximately 30% of the lung tumors propagated i.t. grew in the chest wall and/or in the lung parenchyma as well as in the pleural space. In contrast, tumors propagated i.b. grew predominantly in the lung parenchyma. When five non-pulmonary human tumor cell lines (including U251 glioblastoma, LOX amelamonic melanoma, HT-29 colon adenocarcinoma, OVCAR 3 ovarian adenocarcinoma, and adriamycin-resistant MCF-7 breast adenocarcinoma) were propagated i.b. or i.t., there was considerable site-specific variability in tumor-related mortality depending on the tumor type. These data demonstrate that both the i.b. and i.t. models should be useful for the in vivo propagation and study of certain human pulmonary and nonpulmonary carcinomas as well as being advantageous for future studies of cancer biology and developmental therapeutics.

INTRODUCTION

Recently, there has been increased interest in the use of in vivo models for the propagation of human tumors at organ-specific (orthotopic) sites in athymic nude mice (1–6) and selected orthotopic models for the propagation of renal cell carcinomas (1–4), certain brain tumors (6), colorectal carcinomas (1), and pancreatic carcinomas (5) in immunodeficient mice have been previously described. The conceptual validity of these models, as well as previously described metastatic tumor models (1), was first suggested by the original "seed and soil" hypothesis proposed by Paget in 1889 (7). According to this theory, organ-site specific implantation of tumor cells is essential for optimal growth and progression of tumors in vivo.

A major impediment to the preclinical investigation of human lung cancer, the number one cause of cancer-related adult deaths and a major cause of morbidity and mortality in the U.S. (8–11), has been the absence of optimal in vivo animal models for this disease. Xenograft s.c. models (1, 12, 13) as well as intrarenal implantation methods (2, 14–16) have previously been employed for the study of human lung carcinomas, but these models have not proven entirely satisfactory (1–3, 17, 18). Orthotopic implantation of pulmonary cancers might provide an improved model for propagation and study of these tumors. This concept is supported by a recent report by McLemore, et al., which has described an i.b.² model for propagation of human lung cancers in athymic nude mice (19). The present report describes another approach to the propagation of human lung tumors whereby tumor cells are implanted i.t. in immunodeficient mice and compares this i.t. model with previously described i.b. and s.c. models.

MATERIALS AND METHODS

Human Tumor Cell Lines. The continuous human tumor cell lines employed for these studies have been previously described with regard to their origin, characterization, and maintenance in a recent report by Alley et al. (20). Each cell line was propagated in vitro utilizing standard sterile culture techniques after recovery from cryopreserved seed stock prepared and maintained at the NCI-FCRF tumor repository (19).

Experimental Animals. Female athymic NCr nu/nu mice were obtained from the NCI-FCRF at approximately 4–6 weeks of age and were free of known pathogens at the time of study. All procedures described below were performed in a pathogen-free barrier at NCI-FCRF.

i.b. Implantation. Animals were anesthetized in a Harvard small animal anesthesia chamber (Harvard Biosciences, Boston, MA) employing a 7% flow of nebulized metafane (Pitman Moore, Inc., Washington Crossing, NJ)/100% oxygen mixture. Implantation i.b. was then performed using either 1.0 x 10⁶, 1.0 x 10⁵, or 1.0 x 10⁴ tumor cells, in a 0.1-ml final volume of HBSS (NCI-FCRF Medium Preparation Laboratory) as previously described (19). Animals were subsequently returned to holding cages and observed for tumor development.

i.t. Implantation Procedure. Animals were fully anesthetized as described above and i.t. injections were then performed at the lateral dorsal midaxillary line just below the inferior border of the scapula with a 1.2-cm, 27-gauge needle. The needle was advanced approximately 5 mm through the chest wall into the pleural space, and a tumor cell inoculum of 1.0 x 10⁶, 1.0 x 10⁵, or 1.0 x 10⁴ cells was then dispersed into the pleural space in a final volume of 0.1 ml HBSS (Fig. 1). The procedure requires approximately 1 min for completion and is relatively easy to perform. Prior to return to their holding cages, animals were placed under heat lamps for approximately 10 min to maintain body temperature.

² The abbreviations used are: i.b., intrabronchial; i.t., percutaneous intrathoracic; i.c., intracranial; HBSS, Hanks' balanced salt solution; NCI-FCRF, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD.
s.c. Implantation Procedure. Inocula of $1.0 \times 10^7$, $1.0 \times 10^6$, $1.0 \times 10^5$, or $1.0 \times 10^4$ tumor cells in a 0.1 ml final volume of HBSS, were placed s.c. in anesthetized mice in the lateral ventral midaxillary line with a 27-gauge needle as previously described (19).

Chest Roentgenographic Studies. A Senographe SOOT mammographic device (Thompson-CGR Medical Corporation, Columbia, MD) with a 0.1-mm focal spot with comparable screens, cassettes, and X-ray film (DuPont Lodose System; DuPont, Inc., Trenton, NJ) was employed for roentgenographic studies as previously described (19). Gastrovist® radiopaque contrast material (Berlex Industries, Wayne, NJ) was implanted i.t. or s.c. in a 0.1 ml volume for contrast dye-enhanced radiographic studies (Fig. 1, B and C). Animals were immediately evaluated by anterior-posterior and/or right lateral chest roentgenography following injection of the contrast material.

Monitoring Animals for Tumor Development. Animals that received either i.b., i.t., or s.c. implants were followed daily for signs of tumor development. Those animals exhibiting tumor-related respiratory distress or debilitation from s.c. tumors were killed by CO2 inhalation and all animals not showing clinical signs of tumor within 200 days were killed. The major organs (lung, liver, spleen, kidney, and brain) were removed and fixed in 10% buffered formalin and then processed for histopathological evaluation. Paraffin sections were stained with hematoxylin and eosin and subsequently evaluated for tumor or other histopathology. Animals expiring immediately from postoperative complications and demonstrating no evidence of tumor represented approximately 5% of the animals studied for i.b. implantation and 8% for i.t. implantation; these were eliminated from the tumor-related mortality evaluations and comparisons.

RESULTS

i.t. Implantation. Tumor-bearing animals became progressively cachectic and dyspneic following i.t. implantation and with this technique, tumor cells first grew predominantly in the pleural space and subsequently invaded the lung parenchymal and/or chest wall structures. An overall tumor-related mortality of 92% was observed when the six lung cancer continuous cell lines employed in the present studies were implanted i.t. at a $1.0 \times 10^6$ tumor cell inoculum (Fig. 2). Local mediastinal invasion was observed with the i.t. model in approximately 20% of the tumor-bearing animals. Occasionally distant metastases were also observed for the i.t. implantation method (<1%), with metastases occurring in the left lung, and liver. Local invasion of the right chest wall structures was also noted in approximately 30% of animals following i.t. implantation. This was probably due to deposition of tumor cells in the puncture site on withdrawal of the needle and/or to inadvertent puncture of the lung parenchyma during the i.t. procedure. Histological appearances of tumors obtained from the i.t. implantation model were consistently similar to the parent tumor cell lines from which they were derived.

Comparison of i.b., i.t., and s.c. Models for Propagation of Human Lung Tumor Cell Lines. The i.b., i.t., and s.c. models were compared for efficiency of propagation of human lung tumor cell lines in a parallel study employing the six different continuous human lung tumor cell lines, using three different tumor cell inocula ($N = 184$, i.b., $N = 185$ i.t., $N = 180$ s.c.; Fig. 2). After a $1.0 \times 10^6$ tumor cell inoculum, 100% tumor-related mortality was observed for the i.b. technique and 92% for the i.t. implantation model, whereas minimal (5%) tumor-related mortality was observed when the s.c. tumor model was employed ($P < 0.001$, nonpaired one-tailed, Student's t-test). The low s.c. mortality was most likely related to a lower s.c. tumor propagation efficiency since no surviving s.c.-implanted animals had detectable tumors at the time of necropsy. In addition, when lower ($1.0 \times 10^5$ or $1.0 \times 10^4$) tumor cell inocula were employed, an inoculum size-dependent mortality was observed for both the i.b. and i.t. implantation methods (Fig. 2).

Comparison of the i.b. and i.t. implantation techniques generally demonstrated similar tumor-related mortality when a $1.0 \times 10^6$ tumor cell inoculum was employed (Fig. 2). There was, however, tumor-specific variation observed for the NCI-H358 cell line, in that only 50% tumor-related mortality was observed when these tumor cells were implanted i.t. compared with 100% mortality for the i.b. implantation method at a $1.0 \times 10^6$ tumor cell inoculum (Fig. 2). At $1.0 \times 10^5$ and $1.0 \times 10^4$ inocula, i.b. implantation generally yielded slightly steeper tumor-related mortality than the i.t. method. This was, however, also tumor cell line-dependent as demonstrated by the sharper mortality curve for the i.t. technique when cell lines such as the NCI-H460 and A-549 were employed (Fig. 2).

Histological comparison of tumors obtained from the i.b., i.t., and s.c. models was also performed (Figs. 3 and 4). In the case of s.c.-propagated tumors for the NCI-H460 and NCI-H69 cell lines, a $1.0 \times 10^6$ tumor cell inoculum was required to obtain s.c. tumors for comparison. These comparisons clearly demonstrated that the i.t., i.b., and s.c. models yielded tumors which were characteristic of the original human lung cancer cell lines, at least with regard to histological appearance and...
Fig. 2. Comparison of tumor-related mortality for athymic nude mice implanted i.b., i.t., or s.c. with various human lung carcinoma cell lines. The figure depicts tumor-related mortality/unit time for i.b. (A), i.t. (B) and s.c. (C) implantation of lung tumor cell lines at 1.0 × 10⁶ (A), 1.0 × 10⁷ (B), 1.0 × 10⁸ (C) tumor cell inocula. A minimum of 10 animals were studied for each inoculum size and site. See text for statistical comparisons.

growth characteristics. As previously described (19), i.b. implanted tumors were localized to the lung parenchyma and were predominantly located in the right lung. In contrast, i.t.-implanted tumors were frequently (in approximately 30% of the animals tested) located in the right pleural space, lung parenchyma, as well as in the chest wall.

Roentgenographic techniques were also employed to evaluate human lung tumor growth following implantation using the three different techniques. Radiological comparison of tumors propagated i.b., i.t., and s.c. was performed using a low-dose, high-resolution mammographic radiological device (Figs. 5 and 6). Right-sided lung tumors were easily demonstrated by roentgenography for either the i.b. or i.t. models (Fig. 5). Tumors propagated s.c. were also easily distinguishable by X-ray analysis, but were not as distinct as those tumors arising in the thorax (Fig. 6). This technique was useful for identification of early lesions in both the i.b. and i.t. models when right lateral roentgenographs were employed. Moreover, the progression of these tumors could be estimated radiographically over time (Fig. 5). The potential sensitivity of this method is further illustrated by its ability to define an infiltrate in the right lower lobe of an animal immediately following i.b. implantation. This X-ray finding represents the anatomic location where the tumor cell suspension was i.b. implanted (see Fig. 5 for further discussion).

Comparison of i.b. and i.t. Models for Propagation of Human Nonpulmonary Tumor Cell Lines. Five human nonpulmonary continuous tumor cell lines were also evaluated for their ability to be propagated i.b. or i.t. (Fig. 7). Interestingly, when the U251 cell line was implanted either i.b. or i.t. at a 1.0 × 10⁶ tumor cell inoculum, 100% tumor-related mortality was noted and when this brain tumor was propagated orthotopically (i.e.),

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90% tumor-related mortality was observed. With lower (1.0 × 10^9 or 1.0 × 10^6) tumor cell inocula, an inoculum-dependent mortality was noted for all three implantation routes. Similarly, when the LOX or the HT-29 cell lines were implanted i.b. or i.t. at a 1.0 × 10^6 tumor cell inoculum, high (≥70%) tumor-related mortality was observed, whereas minimal mortality was noted when these cell lines were implanted s.c. at a 1.0 × 10^6 cell inoculum (Fig. 7; P < 0.001). Diminished mortality was
breast carcinoma cell line was implanted i.t. at a $1.0 \times 10^6$ inoculum when the ovarian carcinoma cell line was implanted i.t., but 80% tumor-related mortality was observed when the breast carcinoma cell line was implanted i.t. at a $1.0 \times 10^6$ tumor cell inoculum.

**DISCUSSION**

The present report describes a different model for the in vivo propagation and study of human pulmonary cancers in immunodeficient mice employing an i.t. implantation technique and compares this model with the previously described i.b. model. Both the i.b. and i.t. tumor implantation procedures represent the first relatively short-term animal models for the efficient in vivo propagation of human lung tumors. These techniques are relatively easy to perform, are reproducible, and require a small number of animals and minimal human lung tumor cells for experimentation. These models also offer the advantage of employing human lung tumor cell suspensions rather than relying on less ideal carcinogen-induced animal tumors (21–27) or other nonhuman lung tumors (28). These models potentially allow investigators improved opportunity for study of the growth, progression, metastasis, and experimental therapeutics of human lung tumors in vivo.

A potential disadvantage of the i.t. model is raised by the observation that approximately 30% of all tumors propagated by this method grow in the chest wall as well as in the pleural space and the lung parenchyma. This may limit the usefulness of the i.t. model for selected in vivo drug testing studies and those detailed tumor biology studies which require that the tumor cells grow in a localized intrapulmonary microenvironment. In these instances, the i.b. model might provide a more ideal system for the in vivo investigation of human lung cancers (see Ref. 19 for discussion).

Greater tumor propagation efficiencies were observed for the i.b. and i.t. models compared with the previously described s.c. xenograft model, which requires a $\geq 1.0 \times 10^7$ tumor cell inoculum for optimal tumor propagation efficiency (1, 12, 13, 17), when human lung tumor cell lines were implanted at low ($1.0 \times 10^6$) tumor cell inocula. The decreased tumor-related mortalities associated with s.c. implantation in the present studies appeared to be directly related to the low s.c. tumor propagation efficiencies observed for the tumor cell inocula employed. In addition, certain nonpulmonary tumors such as the U-251 glioblastoma and the LOX melanoma cell lines were very effectively propagated either i.b. or i.t. Interestingly, in the case of the glioblastoma cell line, equally high propagation efficiencies were noted for both implantation of these tumor cells in the lung and for orthotopic (i.c.) implantation. Furthermore, previous studies have demonstrated that certain small cell lung carcinoma cell lines are very effectively propagated i.e. at low ($1.0 \times 10^6$) tumor cell inocula (29, 30). These data,
COMPARISON OF INTRATHORACIC MODELS FOR LUNG CANCER

Fig. 7. Comparison of tumor-related mortality in athymic nude mice implanted i.b., i.t., i.c., or s.c. with various human nonpulmonary tumor cell lines. This figure depicts comparison of tumor-related mortality for five nonpulmonary tumor cell lines implanted i.b. (●), i.t. (▲), i.c. (■), or s.c. (○) at 1.0 × 10⁶ (A), 1.0 × 10⁵ (B), or 1.0 × 10⁴ (C) tumor cell inocula. N = 10 for each implantation site at each inoculum size. See text for statistical analyses.

combined with the present observations, suggest that pulmonary and central nervous system tissues have similar microenvironments which provide support for growth and progression of certain lung as well as brain tumors. Conversely, other nonpulmonary tumors, such as the OVCAR 3, gave relatively low propagation efficiencies when implanted i.b. or i.t.; further supporting the concept that an appropriate organ site-specific microenvironment might be essential for optimal tumor cell growth.

A differential propagation efficiency was also observed between the NCI-H358 lung tumor cell line implanted i.t. versus i.b. The tumor-related mortality was 50% after i.t. implantation compared with 100% when tumor cells were implanted i.b. at a 1.0 × 10⁶ tumor cell inoculum. It is conceivable that the intrapulmonary site offers a selectively advantageous microenvironment for specific lung tumor cell types that is not provided within the pleural space. In this respect, it is also of interest that certain nonpulmonary tumors, including the MCF-7 ADR breast carcinoma cell line, grew more efficiently following i.t. rather than i.b. implantation at a similar tumor cell inoculum.

These data are consistent with the original seed and soil hypothesis proposed by Paget in 1889 (7) and suggest the importance of organ site-specific (although not necessarily orthotopic site-specific) tumor implantation for optimal tumor cell survival and growth. Positive and/or negative selection factors which are present in various mouse tissues appear to be potentially important determinants of the success or failure of human tumor cells to propagate in vivo (1, 31). These factors are apparently important for the established human lung tumor cell lines, including both the small cell and nonsmall cell pulmonary carcinomas, as well as for certain nonpulmonary tumors. A number of tissue characteristics including the abundant blood supply in the lung, the relatively high compliance of lung tissue, and the availability of certain endogenous factors in the lung which support more optimal tumor cell survival and growth (31–38), might all contribute to the enhanced survival and growth of tumor cells in the immunodeficient mouse lung.

The present studies also describe the application of new noninvasive X-ray procedures to periodically monitor lung tumor size for both i.b.- and i.t.-implanted tumors and these may provide an attractive approach for use in experimental therapeutics studies. Implantation of tumor cells into the right lung of nude mice is particularly advantageous since no other major anatomical structures are located in this area which might
interfere with radiographic evaluation of small tumors. Such technology might ultimately provide a two-dimensional, radiographic approximation of tumor size which would allow for a noninvasive in vivo drug testing model which closely resembles the clinical setting in that it makes possible accurate, periodic, noninvasive monitoring of lung tumors following treatment with different therapeutic modalities. It also provides an experimental design whereby each animal serves at its own control.

In summary, this report compares two new models for the in vivo propagation of human pulmonary and nonpulmonary tumor cell lines. These i.b. and i.t. models have specific advantages over other conventional models and should be useful for future in vivo studies of cancer biology and developmental therapeutics.

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