

“Patient-Like” Nude Mouse Metastatic Model of Advanced Human Pleural Cancer

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Abstract Pleural cancer in humans is a frequently occurring tumor. Recently, clinical trials have suggested that chemotherapy and immunotherapy administered intrapleurally may elicit responses in early-stage diseases. However, at radiological and pleural endoscopic evaluation, most of the patients are found to have a visceral pleural involvement that is generally refractory to therapy and leads to a poor prognosis. The goal of this study was to construct a nude mouse model of human parietal- and visceral-pleural cancer that could reflect the clinical picture for this disease. The model could then be useful for drug discovery for pleural cancer. A well-differentiated human lung adenocarcinoma was used as intact tissue for implantation. Ten mice underwent parietal-pleural implantation and ten mice visceral-pleural implantation via a novel thoracotomy procedure we have developed. Symptoms of tumor growth were determined from weight loss, respiratory distress, or debilitation. Actual tumor growth and spread were measured at autopsy. The mouse survival curves of each group were estimated by the Kaplan-Meier method and the difference of the median survival times was assessed by the Log-rank test. The slopes of mean-mouse weight curves were compared using a standard two-sample *t*-test. A 100% take rate was achieved in constructing the pleural cancer models. Tumor growth was initially assessed by symptomatology and survival: the median survival time was, respectively, 27.9 days and 31 days for visceral-pleural and parietal-pleural implanted groups ($P < 0.05$). The comparison between the slopes of the mean weight curves of corresponding groups demonstrated that visceral-pleural implanted animals lost significantly more weight than the parietal-pleural implanted animals ($P < .001$). Both in the visceral- and parietal-pleural implanted groups, post-mortem analysis revealed that tumor grew in all mice demonstrating local and regional spread mimicking clinical features. However, mediastinal lymph node metastases were observed only in mice with visceral pleural implantation. Patient-like models of human parietal-pleural and visceral-pleural cancer were constructed in nude mice using histologically intact human specimens. Tumor symptoms, growth, and spread as well as survival indicated that the parietal-pleural and visceral-pleural models represent, respectively, early- and advanced-stage disease. These “patient-like” nude mouse models of pleural cancer now allow a rational basis for further studies of pleural cancer biology, pathophysiology, and therapeutics. © 1994 Wiley-Liss, Inc.

Key words: nude mouse, pleural model, intact human tumor tissue, pleural implantation

Pleural cancer in humans is a frequently occurring tumor that is generally refractory to therapy [Fentiman, 1987; Hausheer and Yarbrow, 1985; Alberts et al., 1988]. However recent clinical trials have suggested that chemotherapy and immunotherapy administered intrapleurally may elicit responses in early-stage disease [Markman, 1985; Rush et al., 1991; Boutin et al., 1991; Astoul et al., 1993]. The construction of suitable animal models could provide a ration-

al basis for further development of therapeutics for this disease.

Subcutaneous implantation of tumor in nude mice has been widely used for establishing animal models for human cancer research [Kyriazis and Kyriazis, 1980; Sharkey and Fogh, 1984]. However the disadvantages of such a technique are the low take rates, and when the tumor grows subcutaneously, the tumor is often encapsulated and fails to develop regionally and distally [Fidler, 1986, 1990]. Orthotopic implantation of cell suspensions conversely often allows a high take rate of locally growing tumor and subsequent regional and distant metastases

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[Kozlowski et al., 1984; MacLemore et al., 1987; Morikawa et al., 1988]. However, recent studies have indicated that cell suspensions may not express the full metastatic potential of the original tumor [Fu et al., 1991a]. This led to the idea that preserving tumor architecture during orthotopic transplantation could also preserve metastatic potential. In this light we developed surgical techniques for the construction of orthotopic transplant models in nude mice using intact tumor tissue obtained from patients with colon [Fu et al., 1991b], pancreas [Fu et al., 1992], stomach [Furukawa et al., 1993], bladder [Fu et al., 1991a], ovarian [Fu and Hoffman, 1993], and lung cancer [Wang et al., 1992]. Indeed the growth and metastatic patterns of the models developed with surgical orthotopic implantation resembled the clinical pattern.

To construct a surgical orthotopic transplant model for pleural disease, we have developed a thoracotomy procedure through which intact human lung adenocarcinoma tissue was successfully implanted onto the parietal pleura of nude mice. The local and regional spread of the transplant human neoplasm mimicked the clinical picture of early-pleural cancer [Astoul et al., 1994]. The present report describes the development of a model of advanced-pleural cancer whereby intact human tumor tissue is implanted on the visceral pleura in nude mice. These patient-like nude mouse models of parietal- and visceral-pleural cancer which, respectively, mimic early- and advanced-human pleural cancer are described and compared in this report.

MATERIALS AND METHODS

Tissue Procurement

A well-differentiated human lung adenocarcinoma was used at passage-four (tumor AC2572, AntiCancer, Inc, San Diego, CA). The xenograft was obtained initially by orthotopic implantation into nude mice of histologically-intact patient tissue [Wang et al., 1992]. Subsequent tumor growth was obtained by serial subcutaneous implantation up to the fourth generation. An approximate 2 cm diameter tumor was then resected aseptically from the xenograft. Surrounding necrotic and connective tissues were removed, and the remaining tumor tissue was cut into several smaller 1 mm³ sections in preparation for pleural implantation.

Experimental Animals

Twenty five, outbred nude (nu/nu) mice (14 females and 11 males), 3–5 weeks of age, were used (bred at AntiCancer, Inc. under NIH guidelines). They were divided into three groups: five mice were used for subcutaneous implantation (control group), ten mice underwent parietal-pleural implantation, and ten mice visceral-pleural implantation.

Tissue Implantation

Anesthesia. The animals were placed in a Harvard small chamber and then were anesthetized with Isoflurane, an inhalation anesthetic. When the animals were fully anesthetized, they were removed from the chamber and placed in a sterile field. Anesthesia was maintained with an inhalation tube.

Subcutaneous implantation. The mice were placed in the left lateral decubitus position, with the four limbs restrained. Tumor tissue was then implanted subcutaneously at the anterior lateral and low part of the right thoracic wall according to previous reports on preferential subcutaneous sites of human tumor growth in nude mice [Kyriazis and Kyriazis, 1980]. Five pieces of tumor were surgically implanted via a 0.5 cm skin incision. A single layer of 6.0 silk suture was used to close the wound.

Thoracotomy. A small, 1 cm transverse incision was made on the left-lateral chest of the nude mouse via the fourth intercostal space. A small incision provided access to the pleural space, and resulted in total lung collapse.

Pleural implantation. All the mice were placed in the right lateral decubitus position, with all four limbs restrained. Five tumor pieces were implanted onto the left visceral pleura in one half of the animals or the left parietal pleura in the others. This is because the left lung is smaller than the right lung thereby making the loss of lung function after left thoracotomy less than after right-sided procedures.

Visceral pleural implantation. Five tumor pieces were sewn together with a 7-0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung by one suture. Two knots were then made and the remaining suture was cut off. All of the lung tissue was returned into the chest cavity.

Parietal pleural implantation. A sterile 7.0 nylon suture on a cutting needle was in-

serted into the pleural cavity two intercostal spaces below the incision, and removed via the thoracotomy incision. Five pieces of tumor were strung over the needle onto the suture. The needle was then reinserted through the thoracotomy incision and withdrawn two intercostal spaces below, adjacent to the initial point of needle entry. The needle was removed, and a knot was tied between the two free ends of the suture, securing the tumor specimens onto the parietal pleura from below, and onto the intercostal muscles above.

For both pleural procedures, the chest wall incision was closed with a 7.0 nylon suture. If there was any evidence of air leak through the incision, additional sutures were placed until the incision was completely closed. A sterile 3 cc syringe with an attached 25 G11/2 gauge needle (angiocatheter) was then inserted into the pleural cavity. The teflon catheter was threaded over the needle, and the needle was removed to avoid lung injury during reexpansion. Using the attached syringe, air was removed from the closed pleural cavity, and the lung was actively re-inflated. Complete lung reexpansion was verified by observation of an increase in respiratory rate, and visualization of the lung through the chest wall of the mouse. Chest muscles and skin were closed with a single layer of 6.0 silk suture. All procedures were performed with a 7 \times magnification microscope. Approximately four animals could be intrapleurally implanted per hour. There was no operative mortality. All animals recovered rapidly from the intervention, and were returned to their cages in satisfactory condition. Performance status remained stable until shortly before death.

Monitoring Animals for Tumor Growth

Animals were followed daily after surgery and monitored for signs of infection, decreased physical activity, and chest wall invasion. Beginning on day 14 after implantation, mice were weighed twice weekly on day 17, 21, 24, 28, and 31. Mean weights were calculated and recorded on a linear graph. Potential pleural tumor growth was determined from evidence of weight loss, tumor-related respiratory distress or debilitation.

Mice were sacrificed by CO₂ inhalation when moribund or by day 31 after the implantation of tumor tissue. Tumor was harvested immediately after death for gross examination prior to formalin fixation for microscopic tissue examination. Tumor width and length were measured in

order to calculate tumor volume using the formula of a prolate ellipsoid [Geran et al., 1972; Norton and Simon, 1979]. Hematoxylin and eosin stains were performed on histological sections using standard procedures. The lung, mediastinal lymph nodes, liver, kidney, and adrenal gland were also processed for macroscopic and histological examination. Any signs mimicking local, regional, or metastatic spread of human pleural cancer were noted.

Data Analysis

The data were analyzed using a two-stage random-effects regression analysis in which the regression parameters for each individual subject are treated as random variables for the combined analysis. Thus, the data for each mouse were subjected to linear regression to determine regression parameters. The distribution of these parameters for the three subject groups was compared using a two sample *t*-test [Feldman, 1988]. The standard two-sample *t*-test was then used for comparing the mean of the slope for each group and as well as the two parts of the parietal-pleural implanted mouse-weight curve, days 14–24 vs. days 24–31. Mouse survival curves were also estimated by the Kaplan-Meier method. The Log-rank test (Mantel-Haenszel) was used to test for differences between the visceral-pleural implanted mouse survival curve and the parietal-pleural implanted mouse survival curve.

RESULTS

Both in the visceral- and parietal-pleural implanted groups, tumor grew in all ten mice transplanted. The median survival time was 27.9 days for the visceral-pleural implanted group and 31 days for the parietal-pleural implanted group ($P < 0.05$) (Fig. 1). The body weights of pleural-implanted mice decreased from the 14th day until day 31 post transplantation for the mice remaining alive at that time.

The visceral-pleural implanted group had the most weight loss (Fig. 2). On day 24, the mean weight-loss was 4.77 gm (25.5% of the mean body weight on day 14) for the group with visceral-pleural implantation versus 1.4 gm (7.3% of the mean body weight on day 14) for the group with parietal-pleural implantation. The mean weight-curve slope comparison between parietal- and visceral-pleural implanted group was, respectively, -0.29 and -0.44 showing a

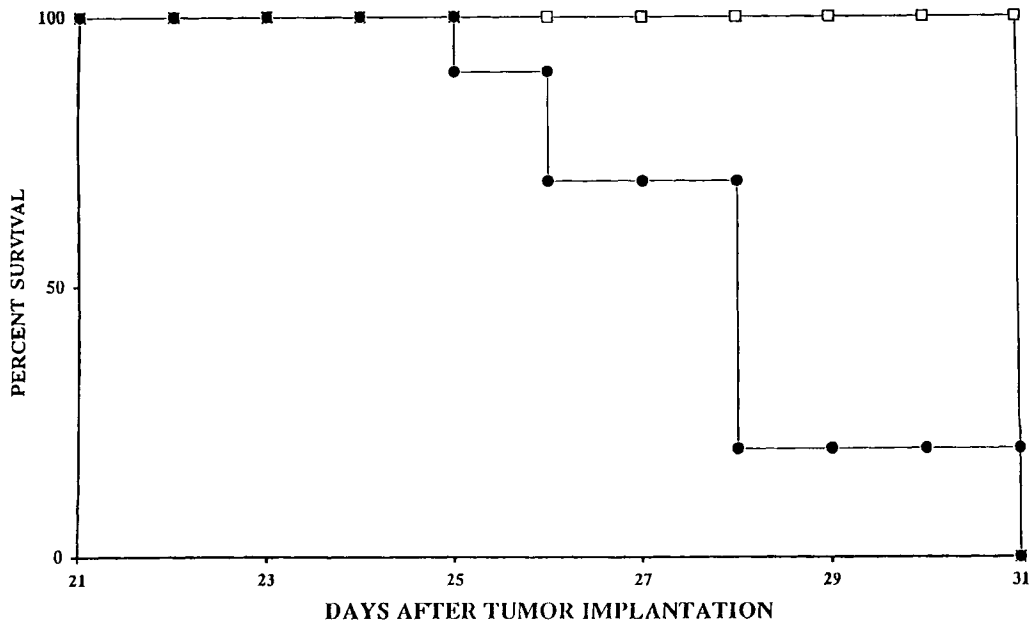


Fig. 1. Survival curves of parietal-pleural implanted mice (□) and visceral-pleural implanted mice (●) with histologically intact human lung adenocarcinoma (id number 2572)

statistically significant difference ($P < .001$). Moreover, the parietal-pleural implanted group showed a steeper decrease of the mean-weight slope curve beginning on day 24. The comparison of the slope between the two parts of this curve, days 14–24 vs. 24–31, was statistically significant ($P < .0001$). The slope of the parietal-pleural implanted mouse curve for days 24–31 was close to the slope obtained for the visceral-pleural implanted group, respectively, -0.49 vs. -0.44 ($P = \text{ns}$).

In contrast no body weight-loss was observed in the subcutaneous implanted group. The mean body-weight was 18.3 gm on day 14 and 18.92 gm on day 31 (slope = 0.04). Pleural tumors for parietal and visceral implantation, respectively, ranged from 104.66 mm³ to 1,884.0 mm³ and from 335.10 mm³ to 1,326.65 mm³ in volume. Mean volumes were respectively, 1,012.65 mm³ (SE \pm 146.85) and 904.59 mm³ (SE \pm 101.74). Histologic examination revealed adenocarcinoma similar to that from which the original tumor specimen was derived.

All pleurally implanted animals had evidence of chest wall invasion. Local and regional spread on macroscopic examination in each pleural implanted group are presented in Table I. Enlarged mediastinal contralateral lymphadenopathies were observed in 5/10 mice with visceral-pleural implantation, but none in the parietal pleural-implanted group. The presence of ipsilat-

eral mediastinal lymph nodes was not determined because of large-tumor involvement of the mediastinum. No metastases were observed in the contralateral lung, liver, kidneys, or adrenal glands in any case. In striking contrast, the subcutaneously implanted animals demonstrated no tumor invasion or spread.

DISCUSSION

Implantation in the posterior and low part of the parietal pleura was chosen because of the presence of pleural stomas previously described [Wang, 1975]. Indeed, such structures are considered to be a gate through which small particles (e.g., malignant cells) are absorbed from the pleural cavity to the lymphatic circulation via sub-mesothelial lymphatic vessels, and also have a connection with sub-peritoneal lymphatics [Wang, 1977; Feldman, 1975]. Furthermore, these stomas are surrounded by macrophages and lymphocytes and are principally located on the inferior part of the mediastinal pleura, on the surface of the diaphragm, and on the lower part of parietal pleura whereas relatively few of them are found in other areas [Wang, 1977]. These structures look like "milky spots" previously described in the peritoneum which are initially infiltrated in the early stages of peritoneal dissemination of cancer [Kanazawa et al., 1970; Hagiwara et al., 1993].

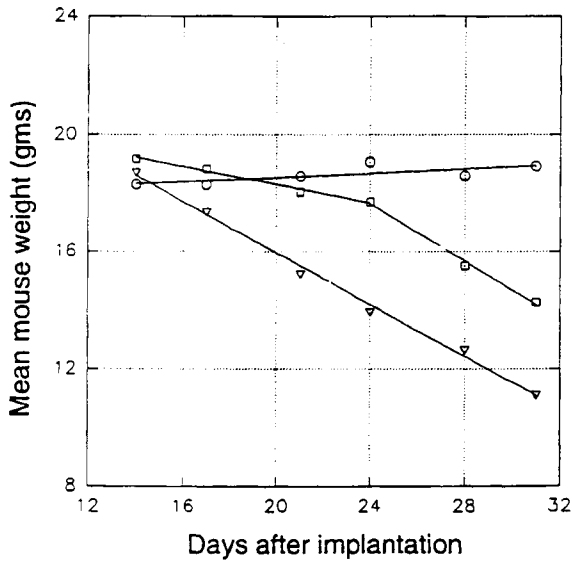


Fig. 2. Weight loss determined from mean body weight curve of human adenocarcinoma tissue-bearing nude mice after direct tissue implantation in pleura. The mean weight for each experimental group was determined for six points in time over 14, 17, 21, 24, 28, and 31. For days 28 and 31, weights of already dead mice were estimated from the individual regression equations before the mean was taken. (○), Subcutaneous implantation; (□), parietal-pleural implantation; (▽), Visceral-pleural implantation.

From clinical observations, it is known that cancers disseminated in the pleural cavity, with the exception of lung cancer progressively involving the ipsilateral pleural cavity, are frequently found in the posterior inferior part of the parietal pleura in early-stage disease. Thus, some authors have speculated that primary pleural cancer develops from the parietal or diaphragmatic pleura and invades the visceral pleura [Canto et al., 1990; Boutin et al., in press]. Such a hypothesis takes into account the difference in prognosis of malignancies localized on the parietal pleura or involving the visceral pleura.

In our study, pleural implantation of tumor leads to symptoms of malignancy, for example, weight-loss in the animals with a significant difference between the parietal and visceral pleural-implanted groups. Moreover, the increase of the slope curve after day 24 in the parietal pleural implanted group leads to the hypothesis of subsequent visceral-pleural invasion. For days 28 and 31, weights of already dead mice were estimated from the individual regression equations before the mean was taken. The random-effects method of analysis was chosen because it allows the effects of the subjects to be weighed equally, notwithstanding the differential mortality (different dates for death). The slope for each subject was based on the measurements taken before its death.

The weight maintenance in the subcutaneous-transplanted group was not related to the volume of the tumor. At autopsy, the mean tumor volume in the parietal pleural-, visceral pleural-, and subcutaneously-implanted group were, respectively, 1,012.65 mm³ (SE ± 146.85), 904.59 mm³ (SE ± 101.74), and 1,561.14 mm³ (SE ± 102.10) which cannot explain the difference in the weight of the host animals. The weight decrease in the pleural-implanted group was not due to mechanical compression of the mediastinum leading to a reduction of activity since clinical symptoms of dyspnea and reduction of the performance status occurred shortly before death.

Although all pleural-implanted animals showed local and regional spread, no macroscopic and microscopic metastases have been seen either on ipsi- or contra-lateral lung in our model as well as in others organs. However, 5/10 visceral-pleural implanted mice developed metastases involving contralateral mediastinal lymph nodes.

TABLE I. Mouse Survival and Loco-Regional Tumor Spread After Subcutaneous, Visceral-Pleural and Parietal-Pleural Implantation of Human Adenocarcinoma Specimens Into Nude Mice as Intact Tissue

	Tumor ^a vol (mm ³)	Tumor spread				Mediastinal lymph node
		Chest wall	Ipsilateral lung	Diaphragm	Mediastinum	
s/c ^b	1561.14	0/5	0/5	0/5	0/5	0/5
pp ^b	1012.65	10/10	9/10	6/10	7/10	0/10
vp ^b	904.59	10/10	10/10	8/10	10/10	5/10

^amean tumor volume: s/c (SE ± 102.10), pp (SE ± 146.85), vp (SE ± 101.74).

^bs/c, pp, vp, respectively, subcutaneous, parietal-, and visceral-pleural implanted group.

We suggest that two probable explanations of the tumor behavior in these models can be advanced: First, the rapid-growth rate of the tumor leads to cachexia and death of the mice before the distant metastases occur. Second, as for peritoneal cancer, we hypothesize that after pleural implantation an inflammatory reaction appears to occlude the stomas connecting the pleural cavity with the submesothelial lymphatic vessels thereby preventing access of the tumor cells to the lymphatic vessels. The presence of pleural effusion in 13 of 20 mice seems to be the indirect result of lymphatic obstruction [Hagiwara et al., 1993]. On the other hand visceral pleural-implanted mice have shown involvement of mediastinal lymph nodes. One possible explanation could be the dissemination of the tumor via the lymphatics vessels localized under the visceral pleura after a period of growth of the implanted tumor.

Thus, visceral pleural involvement represents an advanced-stage disease with respect to greater tumor metastases as well as a shorter mean survival time than observed in the parietal-pleural implanted group which is an early-stage disease. These two components of pleural cancer are mimicked in their respective models described here. Moreover, these models strikingly contrast with the symptom-free survival of subcutaneous-implanted mice.

In summary, visceral-pleural implantation of intact human adenocarcinoma causes weight loss, decreased survival, and as seen at autopsy local and regional tumor growth as well mediastinal lymphadenopathies. The results strongly suggest that the visceral-pleural implanted mice represent an advanced-stage disease, closely resembling the visceral-pleural involvement in human pleural cancer. The parietal-pleural implanted group on the other hand mimics a early-stage pleural cancer. Thus these models reflect the difference of tumor behaviour between human parietal- and visceral-pleural cancer. These "patient-like" nude mouse models of human advanced-stage and early-stage pleural cancer should facilitate further studies of pleural cancer biology, pathophysiology, and lead to new treatment modalities.

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