Clinical Course of Human Epithelial-Type Malignant Pleural Mesothelioma Replicated in an Orthotopic-Transplant Nude Mouse Model

HENRI G. COLT1, PHILIPPE ASTOUL2,3, XIAOEN WANG3, EUNHEE S. YI4, CHRISTIAN BOUTIN2 and ROBERT M. HOFFMAN3,5

1Division of Pulmonary and Critical Care Medicine, 4The Department of Pathology, and 5The Laboratory of Cancer Biology of the University of California San Diego Medical Center, San Diego CA; 3Anti Cancer Inc., San Diego, U.S.A.; 2The Department of Pneumology, Hopital de la Conception, Marseille, France

Abstract. Malignant pleural mesothelioma is an aggressive tumor that is essentially unresponsive to standard medical and surgical therapies. Little is actually known about its biologic response to therapeutic interventions, in part because of a lack of a "patient-like" animal tumor model. Most experimental models thus far have been derived from inhalation or inoculation of asbestos fibers into animal subjects or by subcutaneous transplantation of human mesothelial cell lines into nude mice. These models are not representative of clinical malignant pleural mesothelioma. In this report, an animal model of human pleural malignant mesothelioma obtained by orthotopic transplantation of intact pleural tumor tissue into athymic nude mice is described. Pleural tumor obtained by thoracoscopy from a patient with epithelial-type malignant pleural mesothelioma was implanted as intact tissue by surgical orthotopic implantation (SOI) into the right pleural cavity of nude mice. Animals were sacrificed when moribund or 6 months after implantation. Tumor growth and regional spread in the mice evaluated at post-mortem examination mimicked the clinical pattern of progression of human disease. Histologic findings and the immunohistochemical profile were similar to those demonstrated on examination of thoracoscopic parietal pleural biopsy specimens and post-mortem examination of the original patient's tumor. This "patient-like" nude mouse model of epithelial-type malignant pleural mesothelioma, phenotypically similar to the original human tumor, should facilitate future investigation of tumorigenesis and metastatic potential of this neoplasm. The model should serve as a basis for assessing the impact of experimental and existing therapy on malignant mesothelioma.

Malignant pleural mesothelioma (MPM) is an aggressive tumor that is resistant to radiation, chemotherapy, or surgical resection. Prognosis is poor and death often occurs within two years after the diagnosis. Five year survival is reportedly less than 5% (1,2). Usually, MPM occurs in individuals with a history of asbestos exposure, although at least 20% of tumors may develop spontaneously. Tumor spreads regionally, invading the parietal and visceral pleural surfaces, diaphragm, and mediastinum. Metastatic disease can be found in the contralateral chest and abdomen. Unfortunately, diagnosis is often made late in the course of a patient's illness when medical attention is sought because of chest wall pain, dyspnea, recurrent pleural effusions, or ascites.

Although it appears that malignant mesothelioma develops from a single focus within pleural or peritoneal serosal surfaces, factors influencing cell differentiation and growth are still unclear. Extensive research has resulted in animal models of MPM developed after inoculation or inhalation of asbestos fibers in rats, rabbits, hamsters, and mice (3). Inhalation models are, somewhat "natural", but tumor development, especially in rats, is prolonged, and the small number of tumors that result from fiber inhalation (4) severely limits the use of this model for biologic tumor behavior studies and determining therapeutic responses. MPM developed in rats after intrapleural injection of mineral fibers is artificial, and inconsistently demonstrates specific histopathologic characteristics. Tumor growth may take up to two years (5), and can be unsuccessful despite huge dust loads (6, 7). Subcutaneous transplantation of human MPM in nude mice can lead to tumorigenesis but no metastases. Such ectopic xenograft models are not clinically representative.

Thus, the lack of experimental animal models that...
accurately mimic human disease has made investigation of mechanisms responsible for biologic alterations of the malignant mesothelial cell, tumorigenesis, and metastasis difficult. Orthotopic implantation using histologically intact patient tissue, on the other hand, has repeatedly been shown to produce "patient-like" models of cancer progression and metastasis, and there is no longer any doubt that orthotopic transplantation in the corresponding organ of nude mice results in higher take rates and greater likelihood of metastases than subcutaneous implantation (8, 9).

Building upon our previous experience with "patient-like" nude mouse models of neoplastic disease (10,11), we developed an original rodent model of MPM by orthotopically implanting histologically intact parietal pleural tumor specimens obtained from a patient with MPM onto the parietal pleura of nude mice using surgical procedures termed surgical orthotopic implantation (SOI). Local growth and regional spread of disease closely resembled the clinical pattern of the patient's disease, and all histopathology, including antigen expression measured by immunohistochemistry of nude mouse tumors resembled those of the original human tumor.

Methods

Patient profile. A 39 year old white male with suspected malignant mesothelioma based on substantial asbestos exposure history, chest wall pain, radiographic and computed tomography scan evidence of bilateral pleural thickening, effusions, and volume loss, and pleural fluid cytology was referred for diagnostic thoracoscopic pleuroscopy because of a large, recurrent right-sided pleural effusion and life-threatening respiratory distress. Upon examination, the parietal pleura was diffusely thickened and covered with a white, granular exudate. Clusters of small nodules highly suggestive of malignant mesothelioma were noted on the diaphragm, along the inferior and posterior aspects of the parietal pleura, as well as on the visceral pleura of the upper, middle, and lower lobes. This thoracoscopic appearance was consistent with Bouin stage III malignant mesothelioma (12). Parietal pleura biopsy specimens were obtained and thoracoscopic talc insufflation was performed for pleurodesis. Despite initial improvement of his symptoms, prognosis remained guarded and the patient expired peacefully during his hospitalization.

Histopathology and immunohistochemistry of human pleural tumor. Sections of the pleural biopsy showed a malignant neoplasm composed of epithelioid cells with moderate to abundant eosinophilic cytoplasm, indistinct cell borders, and hyperchromatic nuclei with irregular nuclear contours and prominent nucleoli. The tumor showed a papillary growth pattern at the pleural surface as well as invasive growth into the underlying stroma. There was stromal desmoplastic reaction around the tumor cell nests. No malignant spindle cell proliferation (sarcomatous component) was observed.

On immunohistochemical study, epithelioid tumor cells demonstrated diffuse and strong positive stainings for cytokeratin (Boehringer Mannheim, Indianapolis, IN), vimentin, and epithelial membrane antigen (EMA), but were completely negative for carcinoembryonic antigen (CEA) and Leu M1 (CD 15). All the antibodies were purchased from DAKO (Carpinteria, CA) unless otherwise specified. Negative periodic schiff (PAS) and alcian blue stainings indicated the absence of intracytoplasmic mucin in the tumor cells. The above histologic, immunohistochemical and histochemical findings were consistent with monophasic epithelial type malignant mesothelioma.

Postmortem examination revealed widespread tumor involving bilateral pleura, paratracheal lymph nodes, pericardium, a portion of the left ventricular myocardium, and nearly the entire peritoneum. Tumor was pleural-based and extended into the major and minor fissures as well as into the subpleural parenchyma of the left upper lobe. No nodules or masses were seen within the lung parenchyma distant from the pleural surface, supporting a pleural origin of the tumor. Microscopically, the tumor spread extensively in lymphatic fashion and into the perilymphatic pulmonary parenchyma. The pulmonary and extrapulmonary tumor exhibited features of the epithelioid variant of MPM, identical to the tumor seen in the pleural biopsy. Electron microscopy of pleural tumor tissue obtained at autopsy did not reveal definite long slender microvilli formation or intracellular lumens. Other ultrastructural features frequently seen in MPM were present, however, including basal lamina partially surrounding tumor cells, desmosomes and tight junctions linking individual cells, and abundant cytoplasmic intermediate filaments.

Animals. Athymic four-week old outbred nude mice (nu/nu) were purchased from Charles River Laboratories (Wilmington, USA). Male mice were used for implantation and kept in a sterile, positive pressure room with filtered and humidified air. Cages, bedding, food, and water were autoclaved. Animals were maintained on a daily 12 hour light/12 hour dark cycle. Bethaprida pediatric suspension containing Sulfamethoxazole and trimethoprim was added to the drinking water. NIH guidelines were followed for all animal experimentation.

Tissue preparation. Fresh, human parietal pleura biopsy specimens from areas that appeared abnormal on thorascopic inspection were obtained for histopathologic diagnosis. Discarded fresh tissue specimens destined for implantation were placed in Hank's solution and transported to the laboratory, where they were immersed into ice-cold Earl's minimal essential medium (MEM) containing 70 ml of fetal bovine serum, 5.25 mg/ml of penicillin, 125 mg/ml of streptomycin, 10 ml of fungizone, 5 ml of tetracycline, 50 ml of amikacine, and 75 mg of chloramphenicol per 500 ml. Specimens were trimmed of connective and adipose tissues under sterile conditions and cut into pieces measuring 1-2 mm. Pieces were randomly selected for implantation into each animal in order to enhance reproducibility of tumor growth.

Surgical procedure. One mouse underwent subcutaneous tissue implantation at the anterior and inferior aspect of the right thoracic wall according to previous reports on preferential subcutaneous sites of human tumor growth in nude mice (13). Five pieces of tumor were surgically implanted through a 0.5 cm skin incision, using a single layer of 6.0 silk suture to close the wound.

Orthotopic implantation of tissue into the left pleural space was performed in four mice. Animals were placed in the right lateral decubitus position with all limbs restrained. Anesthesia was administered using Isoflurane. The left lateral chest wall was prepped with iodine and alcohol. A one centimeter thoracotomy incision resulted in collapse of the left lung. A sterile, 8.0 nylon suture on a cutting needle was then inserted into the pleural cavity two intercostal spaces below the initial incision and removed through the thoracotomy incision. Ten, one mm3 pieces of tumor tissue were strung over the needle onto the suture. The needle was reinserted through the thoracotomy incision and withdrawn two intercostal spaces below, adjacent to the point of initial needle entry. The needle was removed and a knot was tied between the two free ends of the suture, securing specimens onto the parietal pleural surface from within the pleural space. The chest wall incision was closed with 7.0 nylon suture.

Residual pneumothorax was evacuated by aspirating the pleural cavity with a sterile 3 cc syringe attached to a 25 gauge 1/2 needle. Chest muscles and skin were approximated using a 7.0 nylon suture. All procedures were performed using a 7x magnification microscope. Each
operation took approximately 15 minutes. All animals were aroused and returned to their cages in satisfactory condition. Postoperative surveillance included twice weekly examination for infection or symptoms suggestive of tumor growth: decreased physical activity, chest wall or subcutaneous invasion, and respiratory distress.

**Histology and immunohistochemistry.** Mice were sacrificed by excess CO2 inhalation when moribund or 180 days after pleural tumor implantation. Complete autopsy was performed to examine the extent of tumor spread as well as the in situ growth. After thorough gross examination, representative sections were taken from ipsilateral and contralateral pleura, lungs, mediastinal lymph nodes, liver, and spleen.

Tissues were fixed in 10% neutral buffered formalin followed by paraffin embedding and hematoxylin and eosin (H&E) staining for histologic examination. Immunohistochemical staining was performed on selected paraffin-embedded tissues using the same primary antibodies that were used in the diagnosis of the original human pleural tumor, including cytokeratin, vimentin, EMA, CEA, and Leu M1 (CD 15). The standard avidin-biotin complex (ABC) method was used with biotinylated secondary antibody and 3,3′-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO) as a chromogen (14).

**Results**

A total of 5 nude mice underwent transplantation. In one, subcutaneous implantation resulted in progressive tumor growth. This mouse is still alive and thriving although the tumor has grown to 10 × 12 mm. Of the four mice undergoing orthotopic implantation, one died rapidly and was eaten by the others, so autopsy could not be performed. In another, death occurred 162 days after implantation. The direct cause of death could not be determined, but gross tumor was noted at the implantation site and on the visceral pleura. Two mice were sacrificed 180 days after implantation. One of them (mouse number 4) had demonstrated decreased performance status and appeared ill. The other (mouse number 5) seemed to be exercising normally. At autopsy, however, both demonstrated tumor growth at the implantation site and along the entire parietal pleura (Figure 1), as well as along visceral, mediastinal, and diaphragmatic pleural surfaces (Figure 2). Gross inspection revealed enlarged ipsilateral and contralateral mediastinal lymphadenopathy, but no distal organ metastases were noted.

**Table I. Correlation of local-regional tumor spread of human malignant mesothelioma in patient and after orthotopic transplantation in nude mice.**

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Implant site</th>
<th>Tumor growth*</th>
<th>Parietal pleura</th>
<th>Visceral pleura</th>
<th>Mediastinal pleura</th>
<th>Diaphragm</th>
<th>Mediastinum</th>
<th>Mediastinal lymph nodes</th>
<th>Follow-up</th>
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<tbody>
<tr>
<td>3</td>
<td>Ortho</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Died day 162</td>
</tr>
<tr>
<td>4</td>
<td>Ortho</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Sacrificed day 180</td>
</tr>
<tr>
<td>5</td>
<td>Ortho</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Sacrificed day 180</td>
</tr>
<tr>
<td>1</td>
<td>S.C.**</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Still alive</td>
</tr>
</tbody>
</table>

* Human malignant pleural mesothelioma tumor was transplanted by surgical orthotopic implantation (SOI) and animals were analyzed at autopsy, as described in the text. (+) Indicates site of tumor growth in both patient and nude mouse. ** Subcutaneous implantation and growth (not seen in patient). Mouse #3 died and was eaten by the others, therefore no tissue was available for analysis.

**Table II. Correlation of patient’s and mouse tumor immunohistochemistry profile.**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cytokeratin</th>
<th>Vimentin</th>
<th>CEA</th>
<th>Leu M1</th>
<th>EMA</th>
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<tbody>
<tr>
<td>Patient's tumor</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Mouse tumor</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* (+) Indicates test positivity in both patient and nude mouse tumor. (-) Indicates test negativity in both patient and nude mouse tumor.

There was a striking resemblance between the course of the patient's tumor and the course of the tumor after SOI in the nude mice (Table I).

Sections of the tumor from the pleura and mediastinal lymph nodes of the mouse showed features that were essentially identical to those found in the original human pleural tumor: malignant epithelioid cells grouped in nests or small clusters associated with a prominent desmoplastic stromal reaction (Figure 3a and 3b). Immunohistochemical profile characterized by positive cytokeratin and vimentin stainings and negative CEA and Leu M1 (CD 15) stainings were also similar to the original parietal pleural biopsy (Table II). EMA immunostaining of the mouse tumor was equivocally positive, unlike the human pleural tumor that demonstrated strong EMA positivity (Table II).

**Discussion**

Malignant pleural mesothelioma is a complex neoplasm that continues to frustrate researchers and clinicians alike. Despite multiple treatment modalities, the prognosis of this disease is grim; death usually occurs from respiratory failure and malignant cachexia within 2 years after diagnosis. MPM most readily occurs in individuals exposed to asbestos, although its onset is often delayed more than 20 years after initial exposure. The incidence rate is increasing at a rate of 13%
Figure 1. Primary tumor (T) growth on left parietal pleura of nude mouse, invading left lung (L) which has become adherent to chest wall.

Figure 2. Macroscopic inspection at autopsy. Gross examination of the right pleural cavity, right lung (rl), heart (H), and diaphragm (d). Tumor has grown through the mediastinum (M), and invaded the ipsilateral and contralateral mediastinal pleura (arrows).
Figure 3. Hematoxylin and eosin stains of human thorascopic pleural biopsy tissue and nude mouse tumor (100 x original magnification). a: Human pleural biopsy shows infiltrative epithelioid cells grouped in variable sized nests (arrows). Exuberant desmoplastic stromal reaction present but without sarcomatoid malignant spindle cell component. Histologic features consistent with monophastic epithelial-type malignant pleural mesothelioma. b: Sections of nude mouse tumor also show epithelioid tumor cell nests (arrows) in desmoplastic stroma, phenotypically identical to the patient’s monophastic epithelial-type malignant pleural mesothelioma.
per year in American men (15): Up to 4000 new cases are expected to be discovered in the United States each year (16).

Definitive diagnosis is difficult, and even after examination of large amounts of tissue, differentiating between bronchogenic adenocarcinoma and the various morphologic types of MPM is not a simple task. Therefore, physicians must additionally rely on clinical history, radiographic appearance, and on a battery of immunohistochemical tests for diagnosis.

In this report, a nude mouse model of MPM derived from orthotopic transplantation of human pleural tumor tissue is described. The patient's clinical history and presentation were suggestive of MPM. At autopsy, the macroscopic growth pattern showed massive spread throughout abdominal and thoracic mesothelial surfaces with direct subpleural extension as well as lymphangitic or vascular spread within the lung parenchyma but without a dominant intrapulmonary mass, further supporting this diagnosis. The histlogic and immunohistochemical profiles of the patient's thoracoscopic parietal pleural tumor biopsy and autopsy specimens were also consistent with MPM. The immunohistochemistry profile, including CD 15 (Leu M1) negativity, was strongly suggestive of MPM. This antibody recognizes an antigen expressed by many adenocarcinomas and virtually never by mesotheliomas. The histlogic features, immunohistochemical profiles, and most importantly, the clinical behavior of mouse-grown tumors mimicked those of the original patient (Table 1). Macroscopic features of local and regional spread found at autopsy resembled those found in the patient. No macroscopic or microscopic evidence of metastases to organs outside of the mediastinum were noted. This too, mimics the natural history of human MPM, since distant metastases have been described in less than 5% of cases (17,18).

Laboratory research of mesothelioma tumorigenesis and therapeutic responses to various agents has been hampered because of a lack of patient-like animal models of MPM. Disadvantages of hamster or rodent models obtained by inhalation or intrapleural injection of asbestos or other fibers, for example, are low take rates, long development times, and the fact that these tumors are not of human origin (3,19,20).

Successful subcutaneous xenografts of MPM has been described (21-23). Histologic characteristics are usually preserved in subcutaneous xenografts (24). Because tumors can be maintained by successive subcutaneous implantation techniques (25,26), xenografts remain a constant source for fresh tissue, and can serve as a basis for studies of chemosensitivity (27-30). Mesothelioma xenografts may also be used to study in vivo chemotherapeutic efficacy (31). Low take rates, encapsulation, and failure of regional or distant metastases, however, have been demonstrated in multiple nonorthotopic cancer models (9,32,33). The subcutaneous tissue model of MPM, therefore, may not be realistic with regard to tumor behavior (25,34). In addition, differences between the rodent subcutaneous and human visceral microenvironments may be radically different, resulting in modified biologic behavior, metastatic potential, or nonclinical drug responses (35).

Orthotopic implantation, which in our case consists of direct transplantation of histologically intact tissue obtained from human malignant tumors onto the corresponding site as that from which the human tissue was derived, results in illness that mimics the original clinical disease pattern (36). Orthotopic implantation also allows patient-like expression of metastatic capability as opposed to orthotopic injection of cell suspensions (37-39). We have previously demonstrated that orthotopic implantation of patient-derived lung and pleural cancer tissues results in a high take rate with rapid local tumor growth and metastases resembling the clinical settings (40,41), and that host organ specificity determines cancer progression (42).

In summary, we describe a novel “patient-like” nude mouse model of MPM obtained by orthotopic xenografting of histologically-intact human tumor into athymic nude mice. Disease progression mimicked the clinical scenario, resulting in local and regional spread similar to that of the original human tumor. The mouse-grown tumor was phenotypically similar to the original human tumor as evidenced by similar histologic features of an epithelial variant of malignant mesothelioma, sites of tumor spread, and immunohistochemical profile. Future investigations using this nude mice model will serve to study the influence of microenvironment on metastatic potential, as well as to stimulate further research of patient-specific pleural tumor responses to cytotoxic therapies and biologic modifiers.

References


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