Cytotoxic Activity of the Recombinant Anti-mesothelin Immunotoxin, SS1(dsFv)PE38, Towards Tumor Cell Lines Established from Ascites of Patients with Peritoneal Mesotheliomas

QIAN LI1, CLAIRE F. VERSCHRAEGEN2, JOHN MENDOZA3 and RAFFIT HASSAN4

1Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK; 2Cancer Research and Treatment Center, The University of New Mexico Health Sciences Center, Albuquerque, NM; 3The Stehlin Foundation, Houston, TX; 4Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.

Abstract. Background: Mesothelin, a cell surface glycoprotein, is an attractive candidate for targeted therapy given its overexpression, as detected by immunohistochemistry, in mesotheliomas. The goal of this study was to evaluate mesothelin expression in fresh tumor cells obtained from ascites of patients with peritoneal mesothelioma, as well as to determine the sensitivity of these cells to an immunotoxin targeting mesothelin. Materials and Methods: Tumor cells were evaluated for mesothelin expression by flow cytometry using the murine anti-mesothelin monoclonal antibody K1. The sensitivity of these tumor cells to SS1(dsFv)PE38, an immunotoxin consisting of the anti-mesothelin Fv linked to a mutated Pseudomonas exotoxin, was evaluated using a cell proliferation assay. Results: Of the 7 tumor cell lines established from ascites of 12 patients with peritoneal mesothelioma, 6 expressed mesothelin while one cell line did not. Cell lines that expressed mesothelin were very sensitive to SS1(dsFv)PE38 with IC50s ranging between 0.08-3.9 ng/ml, while the cell line that was mesothelin-negative was resistant to SS1(dsFv)PE38. Conclusion: High expression of mesothelin is seen on tumor cells of patients with peritoneal mesothelioma and correlates with sensitivity to SS1(dsFv)PE38.

Clinical studies of SS1(dsFv)PE38 in patients with peritoneal mesotheliomas are ongoing.

Malignant mesotheliomas are uncommon tumors, which arise from the serosal cells lining the pleural, peritoneal and pericardial cavities and occasionally from the tunica vaginalis testis (1). The most common anatomical location is the pleural mesothelioma followed by peritoneal mesothelioma. Mesotheliomas involving the pericardium or the tunica vaginalis testis are extremely rare. Out of the approximately 2200 new cases of mesothelioma diagnosed each year in the United States, 10% - 20% are peritoneal mesothelioma (2, 3). The majority of patients with peritoneal mesothelioma have widespread peritoneal dissemination at presentation leading to pain, abdominal distension, ascites and bowel obstruction (4, 5). No effective treatments are available for these patients, resulting in a median survival of less than 12 months (6, 7). Some specialized centers using multimodality treatments including surgery, hyperthermic intra-operative chemotherapy and perioperative intraperitoneal chemotherapy have reported a slightly improved prognosis (8, 9).

Given the poor response of mesotheliomas to standard chemotherapeutic agents, drugs that act by a different mechanism are needed. Examples of such novel agents in clinical trials include angiogenesis inhibitors such as bevacizumab, selective inhibitors of epidermal growth factor receptor tyrosine kinase such as ZD1839, and biologic agents such as intraperitoneal administration of recombinant human interleukin 12 (10, 11). Another therapeutic strategy is the identification of tumor antigens to be used as targets for antibody-based treatments or for the development of tumor vaccines (12). A potentially useful antigen for targeted therapy of mesotheliomas is mesothelin, a 40 kDa glycosylphosphatidylinositol (GPI)-linked cell surface...
Establishment of peritoneal mesothelioma cell lines. Tumor cells were isolated from neoplastic effusions by centrifugation and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal calf serum and 2 mM glutamine. The cells were then plated in tissue culture plates and remained in culture until confluent, before the first tissue culture passage. A cell culture was considered established if it could be carried through at least 5 in vitro passages.

Tumorigenic potential of the peritoneal mesothelioma cell lines. The ability of the peritoneal mesothelioma cell lines established from patient ascites to form tumors was evaluated in nude mice. All cell lines except ROB were evaluated for tumorigenic potential. Nude homozygous mice of Swiss background were injected with 1 x 10^7 tumor cells subcutaneously, and the mice were followed for development of tumors. In mice that formed tumors, the tumors were resected and, under sterile conditions, were broken into small pieces, resuspended in culture media and reimplanted into nude mice that were then followed for tumor development. A portion of these tumors was formalin-fixed and paraffin-embedded and tissue sections from these blocks were stained with H&E and evaluated for morphology. A sample of the tumor was also quick-frozen and stored at -70°C, to be used later for isozyme analysis. The enzymes were extracted by grinding the tumor tissue in a homogenizing media containing 0.01M Tris-HCl, pH 7.5, 0.001M 2-mercaptoethanol and 0.001M EDTA. A portion of the supernate containing all the intracellular material was run on a starch gel to check for mouse versus human nucleoside phosphorylase and lactate dehydrogenase to confirm if the tumors were of human origin.

O15, A431 and A431-K5 cells. O15 is a mesothelin-negative tumor cell line obtained from a patient with ovarian endometrioid carcinoma (22). A431 is a human epidermoid carcinoma cell line, which does not express mesothelin, whereas A431-K5 cells are A431 cells expressing mesothelin by transfection (23). A431 and O15 cells were used as negative control and A431-K5 cells as positive control for detecting mesothelin expression and sensitivity to SSI(dsFv)PE38.

Cell culture. Cell lines obtained from patient specimens as well as O15 cells were grown in modified Eagle medium (MEM) supplemented with 10% fetal bovine serum, non essential amino acids, sodium pyruvate and antibiotic/antimycotics (complete MEM). A431 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM/L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

The A431-K5 cells were grown in Dulbecco’s modified essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2mmol/L glutamine, 100IU/ml penicillin, 100 μg/ml streptomycin and 750 μg/ml of G-418 (geneticin).

Recombinant immunotoxins and antibodies. The anti-mesothelin immunotoxin SSI(dsFv)-PE38 was supplied by NeoPharm Inc. (Lake Forest, IL, USA). BL22, an anti-CD22 immunotoxin, and mab K1 were prepared in the Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD, USA. The immunotoxins in 0.9% saline containing 0.2% human serum albumin were stored at -70°C and thawed at room temperature immediately before use in experiments.

Analysis for mesothelin expression by flow cytometry. Log-phase cultures of the cell lines were harvested into single-cell suspensions.
Figure 1. Morphological examination of peritoneal mesothelioma cell lines in culture. These cell lines were established from ascites obtained from patients with peritoneal mesothelioma. The cells demonstrate features seen in malignant mesothelial cells, including pleomorphism and grouping of cells in irregular clusters. The cells also show nuclear variability and multiple nucleoli (scale bar, 100 microns).
Results

Table I. Clinical characteristics of patients with peritoneal mesothelioma from whom the cell lines were established.

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>M</td>
<td>ascites</td>
</tr>
<tr>
<td>60</td>
<td>M</td>
<td>ascites</td>
</tr>
<tr>
<td>66</td>
<td>M</td>
<td>ascites</td>
</tr>
<tr>
<td>74</td>
<td>M</td>
<td>ascites</td>
</tr>
<tr>
<td>66</td>
<td>M</td>
<td>ascites</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>ascites</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>ascites</td>
</tr>
</tbody>
</table>

Establishment of peritoneal mesothelioma cell lines. Seven cell lines were established from 13 specimens of ascites obtained from 12 patients with peritoneal mesothelioma, epithelioid subtype. The rate of cell line establishment was 54%. The median time to first passage was 8 weeks (range, 6-47 weeks) and all cell cultures were passed in vitro at least five times. Figure 1 illustrates the morphology of these cells in culture, which were reviewed by a pathologist. A homogenous population of cells without any contaminating fibroblasts is seen. Though it is difficult to differentiate malignant mesothelial cells from reactive mesothelial cells, the morphological features were consistent with malignant proliferation. The morphological features suggestive of malignant mesothelial cells included: grouping of the cells in irregular clusters with nuclear variability and multiple irregularly-shaped nucleoli, and the presence of a mixture of smaller and larger mesothelial cells that is seen in malignant mesotheliomas.

The clinical characteristics of the seven patients, from whose ascites the cell lines were established, are shown in Table I. All patients had epithelial malignant peritoneal mesothelioma. This diagnosis was based on histological examination of their original tumor specimens, including immunohistochemical studies by pathologists experienced in the diagnosis of malignant mesotheliomas. The mean age of the patients was 60 years (range 33 to 74 years). Five cell lines were established from ascites obtained from male patients and two cell lines were established from female patients.

Tumorigenic potential of the peritoneal mesothelioma cell lines. Of the six peritoneal mesothelioma cell lines that were injected into nude mice two cell lines, YOU and HAY, formed subcutaneous tumors. In both cases the tumors developed 24 weeks after subcutaneous injection of the cell lines. The HAY and YOU tumors have been repassaged in nude mice 4 and 14 times, respectively. Both tumors were confirmed to be of human origin by isozyme analysis, which showed the presence of human nucleoside phosphorylase and lactate dehydrogenase in the tumor tissue.

Flow cytometric evaluation for mesothelin expression. The anti-mesothelin monoclonal antibody K1 was used to detect mesothelin expression on tumor cells. The relative cell proliferation was analyzed using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI, USA), which is composed of a novel tetrazolium compound that is metabolized by viable cells into a soluble formazan that can be quantitated by reading the optical density at 490 nm (MRX Microplate Reader, The Microtiter Company, Chantilly, VA, USA). Survival was calculated by using the formula: survival (%) = (A/B) x 100, where A is the absorbance of treated cells and B is the absorbance of the control cells. The IC50 is the concentration of the immunotoxin that causes a 50% decrease in cell proliferation compared to control. Each experiment was repeated three times and representative data are presented.
mesothelin, are sensitive to SS1(dsFv)PE38 with an IC50 of 2.0, 0.08, 1.9, 3.9, 0.3 and 2.0 ng/ml, respectively (Figure 2). The IC50 for the three cell lines YOU, PRO and HAY was lower than the 2.0 ng/ml IC50 for A431-K5 cells that express mesothelin while A431 and O15 cells do not (22, 23). As shown in Figure 2, mesothelin expression was seen in six of the seven cell lines established from patient ascites. Only one cell line (PET) did not express mesothelin. This flow cytometry data for mesothelioma expression is in agreement with immunohistochemistry data showing that the majority of epithelial mesotheliomas are mesothelin-positive (15).

**Sensitivity of peritoneal mesothelioma cell lines to SS1(dsFv)PE38**. The sensitivity of the cell lines to SS1(dsFv)PE38 was tested in vitro using growth-inhibition assays. The cells were treated with different concentrations of the immunotoxin for 72 h at 37°C and growth inhibition was measured by determining the optical density (OD490). The IC50 is the concentration of the immunotoxin that causes 50% inhibition of cell growth. As a control for the antigen non-specific activity of Pseudomonas exotoxin in our experiments, we also treated the cells with BL22, an immunotoxin targeting the CD22 antigen not present on mesothelioma cell lines (24), and with the anti-mesothelin monoclonal antibody, mab K1.

Our results show that the peritoneal mesothelioma cell lines ROB, YOU, HAY, HEC, PRO and ORT, which express mesothelin, are sensitive to SS1(dsFv)PE38 with an IC50 of 2.0, 0.08, 1.9, 3.9, 0.3 and 2.0 ng/ml, respectively (Figure 2). The IC50 for the three cell lines YOU, PRO and HAY was lower than the 2.0 ng/ml IC50 for A431-K5 cells that express mesothelin by transfection. In contrast PET, which was negative for mesothelin, was resistant to SS1(dsFv)PE38 with an IC50 of greater than 100 ng/ml. All the mesothelioma cells were resistant to BL22, showing that the cytotoxicity of SS1(dsFv)PE38 was due to specific targeting of mesothelin by SS1(dsFv)PE38. None of the cell lines were sensitive to mab K1, which had no cytotoxicity toward A431-K5 cells either.

A431 cells that are mesothelin-negative were resistant to SS1(dsFv)PE38 with an IC50 greater than 100 ng/ml. Also, the O15 cells obtained from a patient with ovarian endometroid carcinoma were not sensitive to SS1(dsFv)PE38. These results are in agreement with the lack of sensitivity of this cell line to SS1(dsFv)PE38 when grown using the three-dimensional in vitro organotypic culture (22).

**Discussion**

Mesothelin, a 40 kDa cell surface glycoprotein overexpressed in mesotheliomas and several other tumors, has limited expression in normal tissues except the mesothelial cells lining the pleura, peritoneum and pericardium (13, 16). Since the mab K1 by itself does not kill mesothelin-expressing cells, we have focused on arming this antibody as well as other anti-mesothelin Fv’s obtained by phage display with a mutated Pseudomonas exotoxin that mediates cell killing. Several such molecules were developed that showed significant anti-tumor activity against mesothelin-positive tumors (19-21). The compound chosen for clinical development, SS1(dsFv)PE38, is a disulfide stabilized recombinant immunotoxin consisting of the anti-mesothelin Fv fused to a mutated Pseudomonas exotoxin (22). The potential clinical advantages of SS1(dsFv)PE38 include its small size (~63 Kd), high affinity for mesothelin and increased activity.

Most of our preclinical work on anti-mesothelin immunotoxins has involved the use of the cell line, A431-K5, which expresses mesothelin by transfection. Since this model may not accurately reflect the potential activity of SS1(dsFv)PE38 against tumor cells in patients with mesothelioma, we wanted to study its activity directly against human mesothelioma cells. Using ascites obtained from 12 patients with peritoneal mesothelioma, we were able to establish seven cell lines, six of which were positive for mesothelin expression. All six cell lines, which expressed mesothelin, were very sensitive to SS1(dsFv)PE38 with an IC50 ranging from 0.08-3.9 ng/ml. However, the cell line PET, lacking mesothelin expression, was resistant with an IC50 greater than 100 ng/ml. This activity was due to specific targeting of mesothelin since BL22, an immunotoxin that does not bind mesothelin, had no targeting activity against mesothelin-expressing cells. Our results show that the tumor cells in the majority of patients with epithelial peritoneal mesothelioma have very high mesothelin expression and are very sensitive to the anti-mesothelin immunotoxin, SS1(dsFv)PE38. Though we did not test tumor cells obtained from pleural effusions of patients with pleural mesotheliomas, we believe that they would also be sensitive to SS1(dsFv)PE38 given the similarities in the biology of peritoneal and pleural mesotheliomas as well as the fact that the majority of pleural mesotheliomas have high mesothelin expression (15).

The activity of SS1(dsFv)PE38 against human ovarian and lung cancer tumor cells has been studied using an in vitro organotypic and in vivo animal model, respectively. Fresh tumor cells obtained from patients with ovarian cancer were grown in fibroblast-containing collagen gels and treated with SS1(dsFv)PE38 (22). Tumors expressing mesothelin showed a dose-dependent sensitivity to SS1(dsFv)PE38, whereas no antitumor activity was seen in tumors that did not express mesothelin. The activity of SS1(dsFv)PE38 was also evaluated in a mouse experimental lung metastasis model using mesothelin-positive (NCI-H226) and -negative Li et al: Targeted Therapy for Peritoneal Mesotheliomas
Figure 2. Mesothelin expression in different cell lines and sensitivity of these cell lines to recombinant immunotoxins or mab K1. Left panel: Mesothelin expression in the cell lines was measured by flow cytometry using the monoclonal antibody K1. Cells treated with mab K1 are shown in gray while cells treated with the isotype-matched murine IgG antibody are shown by the solid black line. In cell lines that express mesothelin there is increased fluorescence (shift to right) of the mabK1-treated cells. All cell lines except A431, O15 and PET express mesothelin. Right panel: After overnight incubation in 96-well culture plates, cells were treated with immunotoxins or mab K1 and incubated for 72 hours. Growth inhibition was analyzed using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay. The IC₅₀ were calculated. A431, O15 and PET cell lines that were mesothelin-negative were resistant to SS1(dsFv)PE38. All other cell lines that were mesothelin-positive were sensitive to SS1(dsFv)PE38 but resistant to BL22 or mab K1. □=SS1(dsFv)PE38, △=BL22, ○= mab K1 and --- represents IC₅₀.
human lung cancer cell lines (25). SS1(dsFv)PE38 selectively inhibited pulmonary metastases produced by the mesothelin-producing NCI-H226 cells. These results and our results using peritoneal mesothelioma cancer cells demonstrate that human tumor cells expressing mesothelin are very sensitive to SS1(dsFv)PE38.

Since mesothelin is not shed into the bloodstream in significant amounts, is highly expressed in several human tumors and has limited expression on normal tissues except mesothelial cells, it is a good candidate for tumor-specific therapy. Based on our pre-clinical studies demonstrating the anti-tumor activity of SS1(dsFv)PE38, we have initiated Phase I studies in patients with mesothelin-positive tumors, including pleural and peritoneal mesotheliomas. We are evaluating two different schedules of SS1(dsFv)PE38 administration. One involves administration of the drug as a 10-day continuous intravenous infusion while the other trial involves giving the immunotoxin as an intravenous bolus injection every other day for three or six doses (26,27). Both studies are open for patient accrual. Recently a soluble mesothelin-related protein was identified in the sera of patients with ovarian cancer and other tumors (28). This protein does not interfere with SS1(dsFv)PE38 therapy in patients in whom blood levels greater than 300 ng/ml are routinely obtained (our unpublished data). However, this variant of mesothelin could be useful as a marker for early tumor detection and follow-up (29).

In conclusion, our results show that the recombinant immunotoxin SS1(dsFv)PE38, is cytotoxic to human mesothelin-expressing cancer cells supporting the rationale for therapeutic clinical trials in patients with mesothelin-positive tumors.

Acknowledgements

We thank the Stehlin Foundation for Cancer Research, Houston, Texas, U.S.A, for providing the tumor cell lines, which were established there from ascites of patients with peritoneal mesothelioma, and NeoPharm Inc. for providing SS1(dsFv)PE38 for laboratory use. We also thank Ira Pastan for reviewing the manuscript and helpful discussion and Anna Mazzuca and Robb Mann for editorial assistance.

This research was supported in part by a research grant from NeoPharm Inc. and a Career Development Award from the American Society of Clinical Oncology to Dr. Raffit Hassan.

References


Received January 14, 2004
Accepted March 9, 2004