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

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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 IC_{50}s 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.*

Abbreviations: BSA, bovine serum albumin; GPI, glycosylphosphatidylinositol; mab K1, murine anti-mesothelin monoclonal antibody; MEM, modified Eagle medium; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute.

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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

glycoprotein (13). Mesothelin is normally present on mesothelial cells lining the peritoneal, pleural and pericardial cavities and overexpressed in several tumors including mesotheliomas, non-mucinous ovarian cancer, squamous cell carcinomas and pancreatic cancer (14-18). Mesothelin is a good target for tumor-specific therapy because it is not present in normal tissues except mesothelial cells and it is not shed into the bloodstream in significant amounts (16).

Since the murine anti-mesothelin monoclonal antibody (mab K1) by itself does not kill mesothelin-expressing cells, we have developed several immunotoxins targeting mesothelin, with the goal of developing the most active agent for clinical use (19, 20). SS1(dsFv)PE38 is a disulfide stabilized recombinant immunotoxin consisting of the anti-mesothelin Fv fused to a mutated *Pseudomonas* exotoxin (21). SS1(dsFv)PE38 has significant *in vitro* and *in vivo* antitumor activity against cells expressing mesothelin by transfection as well as cytotoxic activity against malignant cells of patients with ovarian cancer grown in short-term culture (22).

Though mesothelin expression is seen by immuno-histochemistry in the majority of patients with epitheloid mesothelioma, its expression in fresh tumor cells from patients has not been studied. The goals of this study were to evaluate mesothelin positivity in tumor cells obtained from ascites of patients with peritoneal mesothelioma, as well as to test the sensitivity of these cells to the anti-mesothelin immunotoxin SS1(dsFv)PE38. We chose to use fresh tumor cell lines obtained directly from patients rather than established cell lines because expression of some cell surface antigens decreases in established cell lines. This appears to be the case for mesothelin. The ovarian cancer cell line, OVCAR-3 has about 36,000 sites/cell of mesothelin, which is much less than the 2.5 x 10^6 sites on A431-K5 cells (a human epidermoid carcinoma cell line expressing mesothelin by transfection). The antigen expression on A431-K5 cells more closely approximates that seen on immunohistochemical examination of tumor specimens (23). Therefore, use of fresh tumor samples from patients may be more appropriate to test the activity of compounds targeting mesothelin.

Materials and Methods

Patient specimens. Ascitic fluid was obtained from 12 patients with malignant peritoneal mesothelioma undergoing treatment at the M.D. Anderson Cancer Center, Houston, Texas, USA. The Institutional Review Boards of the M.D. Anderson Cancer Center as well as the Stehlin Foundation for Cancer Research approved the protocol for tissue collection and processing. Ascitic fluid was collected from patients undergoing therapeutic paracenteses. The ascites that was not needed for patient care (approximately 1000 ml per patient) was sent to the Stehlin Foundation Research Laboratory for the establishment of cell cultures.

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 reinjected 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 -80˚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 SS1(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, 2mmol/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 SS1(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

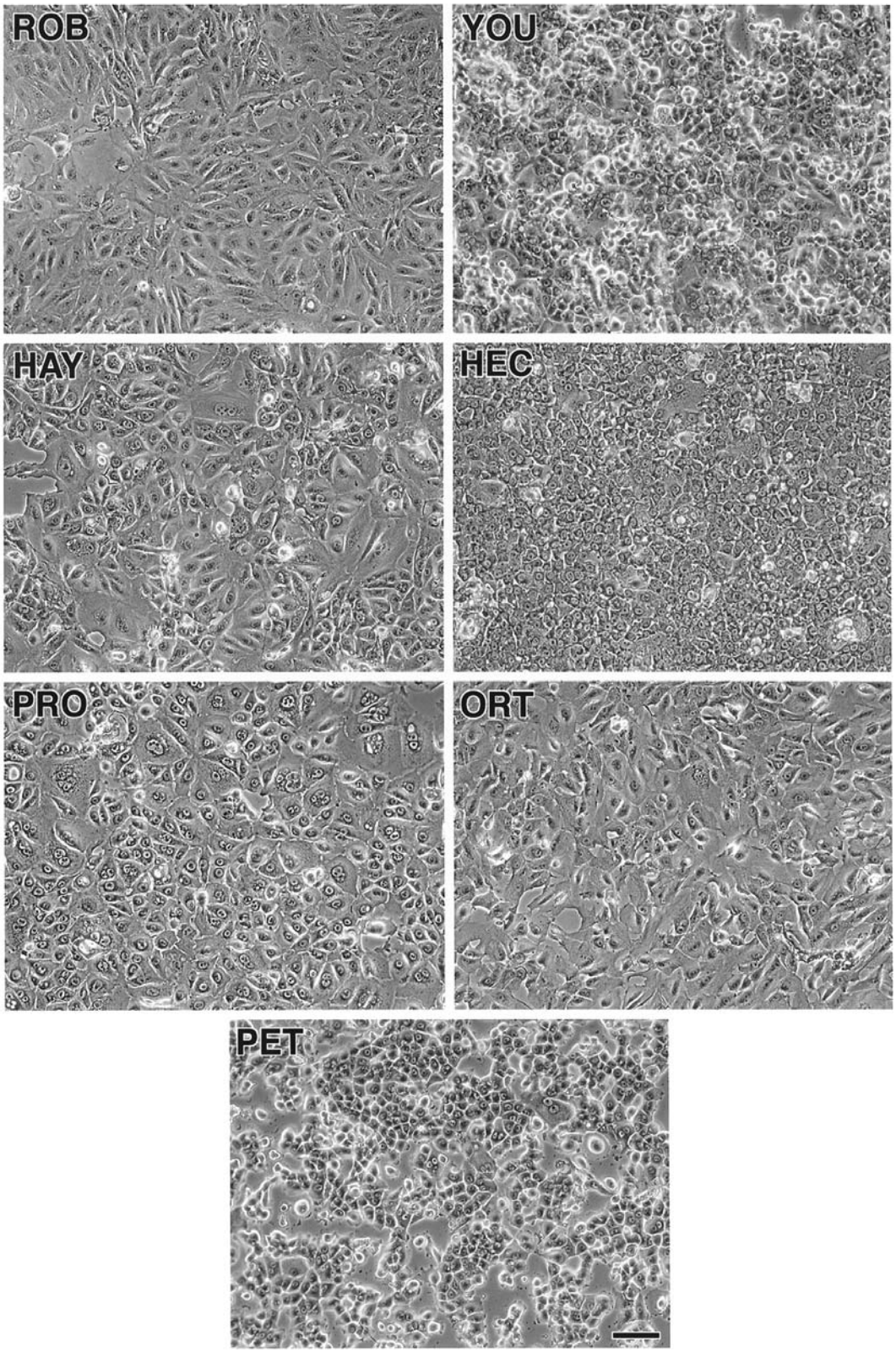


Figure 1. *Morphological examination of peritoneal mesothelioma cell lines in culture. These cell lines were established from ascites obtained from patients with peritoneal mesothelioma. The cells demonstrate features seen in malignant mesothelial cells, including pleomorphism and grouping of cells in irregular clusters. The cells also show nuclear variability and multiple nucleoli (scale bar, 100 microns).*

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

	Age	Sex	Source
ROB	54	M	ascites
YOU	60	M	ascites
HAY	66	M	ascites
HEC	74	M	ascites
PRO	66	M	ascites
ORT	65	F	ascites
PET	33	F	ascites

by brief trypsinization. The cells were washed once with medium and once with ice-cold phosphate-buffered saline (PBS) and then resuspended in ice-cold 1% bovine serum albumin (BSA) in PBS at 300,000 cells/ml in two separate microcentrifuge tubes. In the first tube the cells were incubated with 5 μg/ml of mab K1 and in the second tube with 5 μg/ml of an isotype-matched mouse IgG antibody (Sigma, St. Louis, MO, USA), to serve as a control for the non-specific binding of mouse IgG antibody to the cells, for 1 h at 4˚C. The samples were then washed twice with ice-cold 1% BSA in PBS and incubated with 5 μg/ml of a goat anti-mouse FITC-labeled $F(ab')_2$ secondary antibody (Oncogen, San Diego, CA, USA) for 45 min at 4˚C. The cells were then washed with PBS prior to analysis using a FACSCalibur flow cytometer (BD Biosciences, Bedford, MA, USA).

In vitro cytotoxicity assay. Cells to be evaluated for immunotoxin sensitivity were plated in 96-well microtiter plates at a density of 3000 cells/well. After overnight incubation the media was removed and replaced with medium containing different concentrations of SS1(dsFv)-PE38, BL22 or mab K1. BL22, which consists of the anti-CD22 Fv linked to PE38, was used as a control for the non-specific toxicity of *Pseudomonas* exotoxin, since BL22 does not bind mesothelin. We also treated cells with the unconjugated anti-mesothelin mab K1 to determine if it had cytotoxic activity towards mesothelin-expressing tumor cells. The cells were incubated with the immunotoxins or antibody for 72 h at 37˚C. The relative cell proliferation was analyzed using the Cell Titer 96® A_{queous} 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 IC_{50} 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.

Results

Establishment of peritoneal mesothelioma cell lines. Seven cell lines were established from 13 specimens of ascites obtained from 12 patients with peritoneal mesothelioma, epitheloid 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 using flow cytometry. As a control for the non-specific binding of the murine antibody to the cells, we also treated the cells with an isotype-matched IgG mouse antibody. In Figure 2 the cells treated with mab K1 followed by the FTIC-labeled $F(ab')_2$ goat anti-mouse antibody are shown in gray while the cells treated with the isotype-matched IgG murine antibody followed by FTIC-labeled $F(ab')_2$ goat anti-mouse antibody are shown by the solid black line. In cell lines that are mesothelin-positive, the

mab K1-treated cells have increased fluorescence with shift to the right. There is no difference in the fluorescence between mabK1 and isotype-matched IgG mouse antibody-treated cells in cell lines lacking mesothelin expression.

In concordance with our previous experiments in which mesothelin expression was determined by immunofloresence or immunohistochemistry, flow cytometry results showed that A431-K5 cells 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 mesothelin expression is in agreement with immunohistochemistry data showing that the majority of epitheloid 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(OD_{490}). The IC_{50} 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 IC_{50} of 2.0, 0.08, 1.9, 3.9, 0.3 and 2.0 ng/ml, respectively (Figure 2). The IC_{50} for the three cell lines YOU, PRO and HAY was lower than the 2.0 ng/ml IC_{50} 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 IC_{50} 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 IC_{50} greater than 100 ng/ml. Also, the O15 cells obtained from a patient with ovarian endometrioid 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

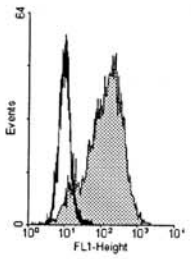
Mesothelin, a 40 kDa cell surface glycoprotein overexpressed in mesotheliomas and several other tumors, has limited expression in normal tissues except the mesothelial cells lining the pleura, peritoneum and pericardium (13, 16). Since the mab K1 by itself does not kill mesothelin-expressing cells, we 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.

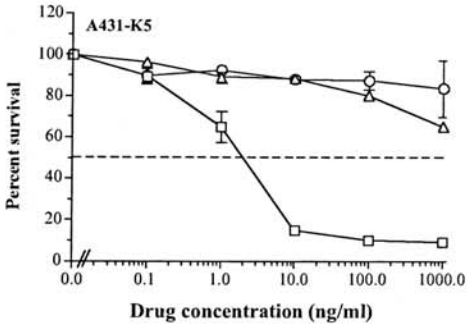
Most of our preclinical work on anti-mesothelin immunotoxins has involved the use of the cell line, A431-K5, which expresses mesothelin by transfection. Since this model may not accurately reflect the potential activity of SS1(dsFv)PE38 against tumor cells in patients with mesothelioma, we wanted to 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 IC_{50} ranging from 0.08-3.9 ng/ml. However, the cell line PET, lacking mesothelin expression, was resistant with an IC_{50} 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

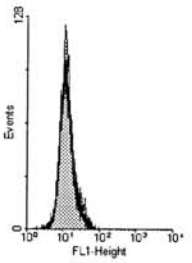
2A

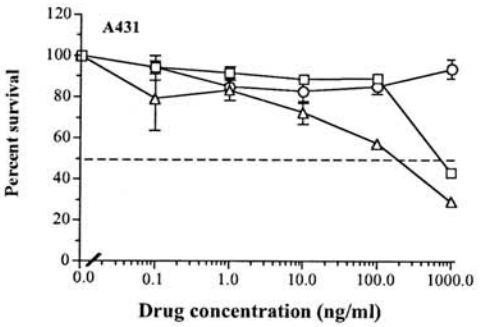
A431-K5



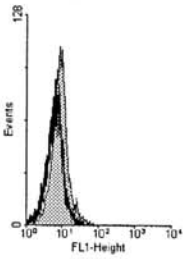


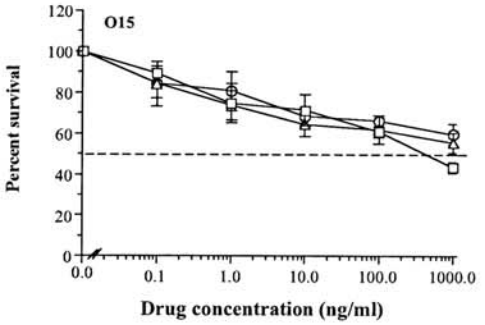
A431



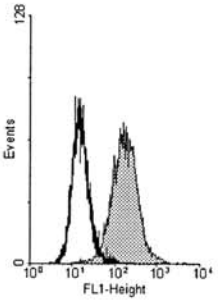


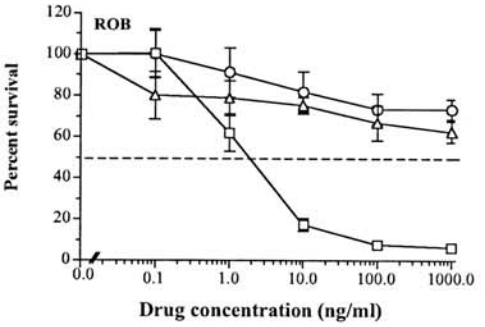
O15



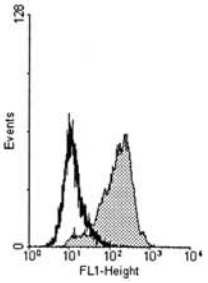


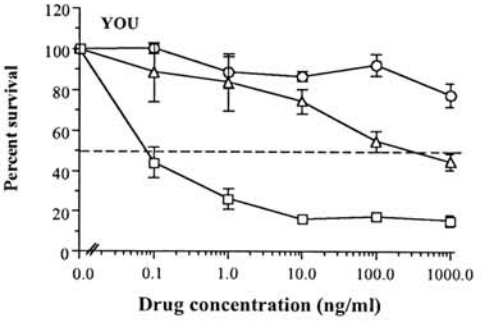
ROB





YOU





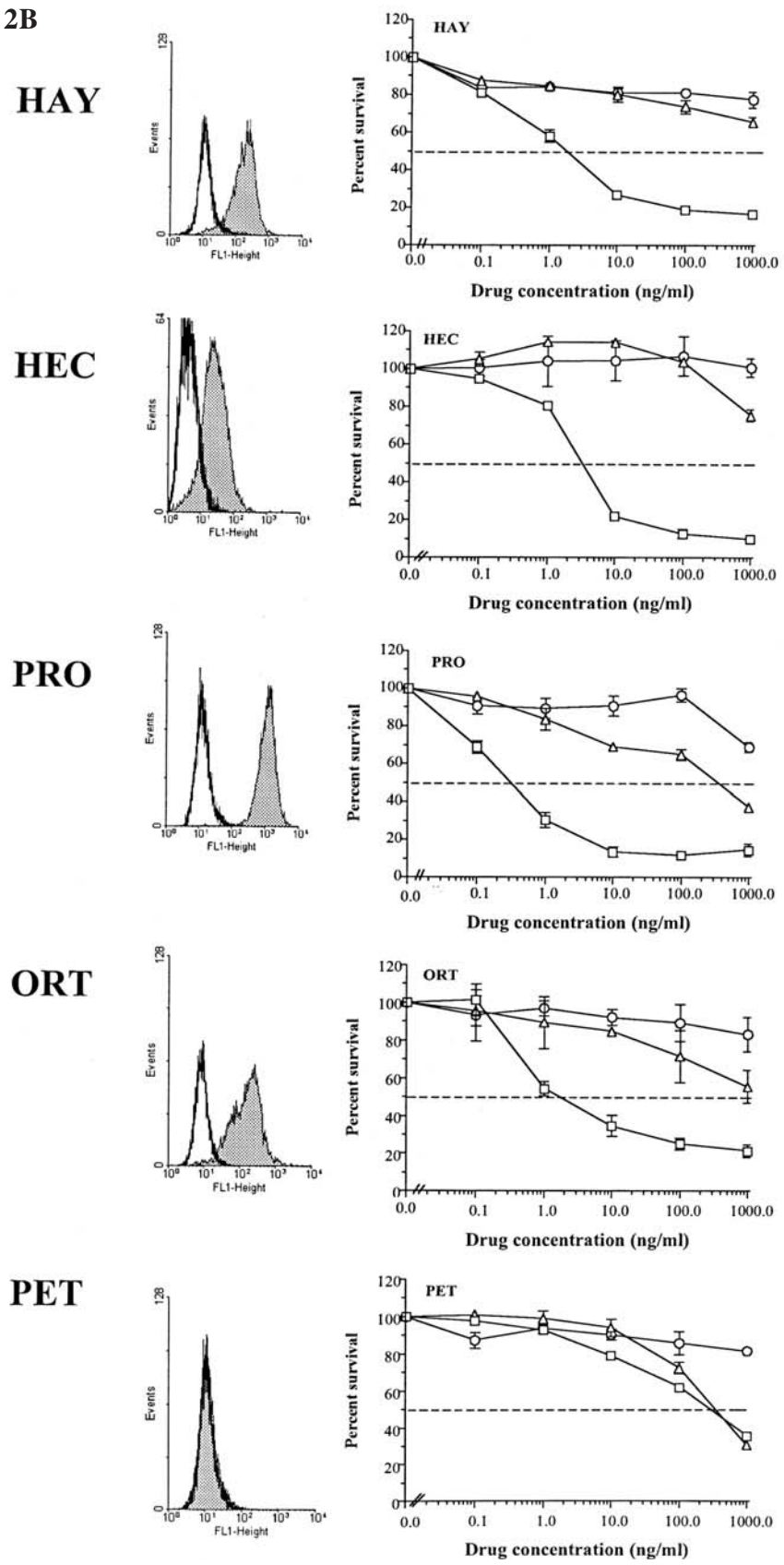


Figure 2. *Mesothelin expression in different cell lines and sensitivity of these cell lines to recombinant immunotoxins or mab K1. Left panel: Mesothelin expression in the cell lines was measured by flow cytometry using the monoclonal antibody K1. Cells treated with mab K1 are shown in gray while cells treated with the isotype-matched murine IgG antibody are shown by the solid black line. In cell lines that express mesothelin there is increased flouresence (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® A_{queous} Non-Radioactive Cell Proliferation Assay. The IC_{50}s 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 IC_{50}.*

(PC14PE6) 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

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