Targeted Therapy against Human Lung Cancer in Nude Mice by High-Affinity Recombinant Antimesothelin Single-Chain Fv Immunotoxin


Abstract
Several tumors, including mesothelioma and ovarian cancer, can overexpress mesothelin, a glycosylphosphatidylinositol-linked differentiation glycoprotein. The membrane-bound type of mesothelin is found in the blood of cancer patients at a very low level, which makes mesothelin a good candidate for targeted therapy of certain cancers. An antimesothelin disulfide-linked Fv (SS1 Fv) was fused to a truncated mutant of Pseudomonas exotoxin A to produce the recombinant immunotoxin SS1(dsFv)-PE38, which has a high binding affinity to mesothelin (Kd = 0.7 nM). Our studies in vitro showed that SS1(dsFv)-PE38 is significantly more cytotoxic to the high-mesothelin-producing NCI-H226 human non-small cell lung cancer cells than to human lung adenocarcinoma PC14PE6 cells, which do not express mesothelin. When administered at a nontoxic dose of 500 µg/kg on days 7, 9, and 11 to nude mice injected i.v. with the two human lung cancer cell lines, SS1(dsFv)-PE38 selectively inhibited experimental lung metastases produced by the mesothelin-producing NCI-H226 cells. Our data indicate that mesothelin-producing squamous cell carcinoma of the lung may be a good target for this immunotoxin.

Introduction
Several differentiation antigens such as CD19, CD20, CD22, and CD25 (1–4) that are often preferentially expressed in cancer cells have been targets for therapy in hematopoietic malignancies. Targeted toxin molecules have been constructed, and their antitumor activity has been demonstrated (5–8), and several immunotoxins are in clinical trials (5, 9, 10). One candidate differentiation antigen, mesothelin, is a GPI-linked glycoprotein synthesized as a M, 69,000 precur- sor; it is proteolytically processed into a M, 30,000 NH2-terminal secreted form and a M, 40,000 membrane-bound form (11). The M, 30,000 secreted form of mesothelin has been termed megakaryocyte-potentiating factor (12, 13). Mesothelin is present only on normal mesothelial cells and on the surface of several tumors, including mesothelioma and ovarian cancer (11, 14, 15). It is not required for normal mouse development or reproduction (16). Its membrane-bound form can be detected in the blood of cancer patients at very low levels, levels too low to interfere with antibody-based therapies (4, 14).

The first antimesothelin monoclonal antibody, K1, was isolated from mice immunized with the ovarian cancer cell line OVCAR3 (15). Although K1 chemically conjugated to a truncated form of Pseudomonas exotoxin binds mesothelin-positive cells and cancer cells, it was not useful as an immunotoxin due to poor internalization of the complex (14). Using a phage display library made from spleen mRNA of mice immunized with recombinant mesothelin produced in Escherichia coli, an antimesothelin scFv was isolated (17). However, this scFv had a low affinity to mesothelin-positive cells and thus was ineffective for therapeutic targeting. Subsequently, immunization with DNA (18) has been used to immunize mice with a mesothelin expression plasmid (19). A scFv, SS1(scFv)-PE38, was isolated from the splenic RNA of those mice using phage display technology (19). The affinity of this Fv was then improved using a different type of phage display. The resulting Fv was used to construct an immunotoxin by genetically fusing it to a truncated mutant of Pseudomonas exotoxin A (20). The purified immunotoxin [SS1(scFv)-PE38] has a high binding affinity to mesothelin, with a dissociation constant (Kd) of 0.7 nM. SS1(dsFv)-PE38 is a stabilized form of the Fv in which a disulfide bond connects the light and heavy chain domains of the Fv (20, 21). It is stable at 37°C for up to 40 h. It is cytotoxic to mesothelin-expressing cells in vitro and produces antitumor effects against ectopic s.c. cervical epithelial carcinoma cells that have been stably transfected with the full-length mesothelin cDNA, pcD3CAK1-9 (19).

Our aim was to test this new therapeutic against lung cancer, the leading cause of cancer death for both men and
women in the United States. During the year 2001, it was predicted that there would be about 169,500 new cases of lung cancer and that about 157,400 people would die of it (about 90,100 men and 67,300 women). More people die of lung cancer than of colon, breast, and prostate cancers combined (22, 23). We hypothesized that tumor cells that express mesothelin on their surface are susceptible to the SS1(dsFv)-PE38 immunotoxin. In this study we used two different human lung cancer cells that differ in expression of mesothelin. Since the outcome of preclinical cancer therapy depends on the use of orthotopic models (8, 24, 25), we injected the cells i.v. to produce lung lesions. We report that SS1(dsFv)-PE38 immunotoxin inhibited experimental lung metastases produced by mesothelin-expressing human lung cancer cells in nude mice.

Materials and Methods

Cell Line and Culture Conditions. The NCI-H226 human NSCLC cell line of the squamous type (26) was a gift of Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). The PC14PE6 cell line (27) was isolated from pleural effusions that appeared in a nude mouse injected i.v. with cells from the heterogeneous human lung adenocarcinoma cell line PC14 (obtained from Dr. N. Saijo; National Cancer Center Research Institute, Tokyo, Japan). Karyotypic analysis of the PC14PE6 cell line revealed that it was highly contaminated with murine cells. All tumor cell lines were maintained as adherent monolayer cultures in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), l-glutamine, pyruvate, nonessential amino acids, 2-fold vitamins, and penicillin-streptomycin (Invitrogen, Carlsbad, CA) and incubated in 6.4% CO2 with balance of air at 37°C.

Preparation of Immunotoxins. The immunotoxins SS1(dsFv)-PE38 and anti-Tac(dsFv)-PE38 were constructed by using PCR-based procedures as described previously (20). Active monomeric protein was purified from the refolding solution by ion exchange and size exclusion chromatography to near homogeneity as described previously (3, 19, 21). Protein concentrations were determined by the Bradford method (Coomassie Plus; Pierce, Rockford, IL).

Flow Cytometric Analysis for Expression of Mesothelin. Log-phase cultures of NCI-H226 and PC14PE6 cell lines were harvested into single-cell suspensions by brief trypsinization. The cells were washed once with medium and once with ice-cold PBS, fixed with 70% methanol at −20°C for 7 min, and then washed with ice-cold PBS. The cells were resuspended in ice-cold 1% BSA in PBS at 1,000,000 cells/ml and then incubated for 45 min at 4°C in the presence of the K1 antibody (15) at 5 μg/ml with intermittent agitation. Cells incubated with the second antibody alone served as negative control. The samples were then washed twice with ice-cold 1% BSA in PBS, reacted with a goat antimouse FITC-labeled F(ab’)2 second antibody (Oncogene Research Products, Boston, MA), and washed with PBS before analysis with an EPICS Profile flow cytometer (Beckman Coulter, Inc., Fullerton, CA; Ref. 28).

Immunofluorescence Staining for Mesothelin Expression. NCI-H226 and PC14PE6 cells were plated on sterile Falcon culture slides (BD Bioscience-Discovery Labware, Bedford, MA) in complete Eagle’s minimum essential medium containing 10% fetal bovine serum. After 48 h, the slides were washed three times with PBS, fixed in cold acetone for 5 min, and incubated with a protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature. The blocking solution was removed, and the primary K1 antibody (15) was added to the cells at a 1:500 dilution. The immunoreaction was allowed to proceed at 4°C for 18 h. After rinsing four times with PBS and incubating the cells in protein-blocking solution for 10 min, we treated the slides with the appropriate dilution (1:200) of secondary goat antimouse antibody conjugated to Texas Red. The slides were washed twice with PBS containing 0.1% Brij and washed with PBS for 5 min. Immunofluorescence microscopy was performed using a ×40 Plan-Neofluar objective (Carl Zeiss, Inc., Thornwood, NY) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel to select for green, red, and blue fluorescence. The expression of mesothelin was identified by red fluorescence, and the images were captured using a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom). Images were further processed using Adobe Photoshop (Adobe Systems, Mountain View, CA) on a computer.

In Vitro Cytotoxicity Assay. Because seeding density can influence the results of a cytotoxicity assay, we first determined the optimal density at which to measure growth inhibition of the cell lines, and we found it to be 1200 cells/well for NCI-H226 and 600 cells/well for PC14PE6 cells. After 4 days of growth, this plating density did not produce con-
fluence, thus allowing for ready quantitation. In all assays, the tumor cells were seeded in 38-mm² wells of flat-bottomed 96-well plates in triplicates and allowed to adhere overnight. The cultures were then washed and refed with medium (negative control) or medium containing various concentrations of the immunotoxins. After 96 h, the antiproliferative activity was determined by the MTT assay (29), which monitors the number of metabolically active cells. After a 2–4-h incubation in medium containing 0.42 mg/ml MTT, the cells were lysed in 100 μl of DMSO. The conversion of MTT to formazan by metabolically active viable cells was monitored by a CERES UV900C 96-well microtiter plate scanner at 570 nm (Bio-Tek Instruments, Inc., Winooski, VT). Survival was calculated from the following formula: survival (%) = (A/B) × 100, where A is the absorbance of treated cells, and B is the absorbance of the control cells.

Experimental Pulmonary Metastasis. To prepare tumor cells for inoculation, we harvested log-phase monolayer cultures of NCI-H226 human NSCLC cells by a 2-min exposure to 0.25% trypsin in a 0.1% EDTA/PBS solution. The cells were pipetted gently to produce a single-cell suspension, neutralized with growth medium, washed, and resuspended in calcium- and magnesium-free HBSS. Log-phase monolayer cultures of PC14PE6 human lung adenocarcinoma cells were harvested by repeated pipetting. Cell viability was determined by the trypan blue exclusion method, and only single-cell suspensions of >90% viability were used for the in vivo studies. To produce experimental pulmonary metastases, 2.5 × 10⁵ NCI-H226 or 1 × 10⁵ PC14PE6 cells in 0.2 ml of HBSS were injected into the lateral tail vein of anesthetized nude mice (30). The i.v. injections of immunotoxins (or control preparations) were administered on days 7, 9, and 11 after tumor cell injection. The mice were euthanized with methoxyflurane when animals in the control group became moribund. The chest wall was resected, and the lungs were removed, weighed, and fixed in Bouin’s solution. Tumor lesions were counted under a dissecting microscope.

Results
Expression of Mesothelin in Target Cell Lines. In the first set of experiments, we measured the level of mesothelin expression in several human lung cancer cell lines to select the appropriate target cell line. The levels of mesothelin expression in RFUs are as follows: NCI-H226 squamous cell carcinoma, 134 RFU; NCI-H322 NSCLC, 67 RFU; NCI-H460 large cell lung cancer, 35 RFU; and NCI-H522 NSCLC, 74 RFU. These data indicated that human lung cancers do express mesothelin at a high incidence. We have chosen to use NCI-H226 in this study because of its higher expression of mesothelin. In subsequent experiments, we compared the expression levels of mesothelin between the NCI-H226 human NSCLC cells and the PC14PE6 human lung adenocarcinoma pleural effusion cells. The antimesothelin antibody K1, which was isolated from mice immunized with the ovarian cancer cell line OVCAR3, reacted with 99.5% of NCI-H226 cells and 0% of PC14PE6 cells as determined by flow cytometry (Fig. 1). The difference in mesothelin expression levels of the two cell lines was confirmed by fluorescent immunohistochemical staining using the K1 antimesothelin antibody and Texas Red (Fig. 2).

Different in Vitro Antiproliferative Effect of Immunotoxins. In the next set of experiments, we tested the effects of SS1(dsFv)-PE38 (antimesothelin immunotoxin) and anti-Tac(dsFv)-PE38 (control immunotoxin) on the NCI-H226 and PC14PE6 human lung cancer cells. Anti-Tac(dsFv)-PE38 was used as a control because it only binds to CD25 and only kills CD25-expressing cells (21). The cells were treated with various concentrations of the immunotoxins for 96 h. Cultures of the mesothelin-expressing NCI-H226 cell line were sensitive to the SS1(dsFv)-PE38 (IC₅₀ = 0.008 ± 0.003 ng/ml, mean ± SE; n = 3), but not to the anti-Tac(dsFv)-PE38 immunotoxin (IC₅₀ > 100 ng/ml; Fig. 3A). The PC14PE6 cells, which do not express mesothelin, did not respond to either immunotoxin (IC₅₀ ≥ 100 ng/ml; Fig. 3B).

Therapeutic Effects of Antimesothelin Immunotoxin. Next, we determined whether SS1(dsFv)-PE38 had a therapeutic effect against experimental lung metastases produced by the NCI-H226 and PC14PE6 human lung cancer cells in nude mice. Groups of mice (n = 10) were given i.v. injections of NCI-H226 (2.5 × 10⁵) or PC14PE6 (1 × 10⁵) cells on day 0. After the tumors were allowed to establish, mice were treated with i.v. administrations of PBS, anti-Tac(dsFv)-PE38 control immunotoxin (10 μg/0.2 ml/mouse), or SS1(dsFv)-PE38 anti-mesothelin immunotoxin (10 μg/0.2 ml/mouse) on days 7, 9, and 11. The tumors of mice injected with the PC14PE6 cells (low-mesothelin-expressing cells) did not respond to either immunotoxin. The tumors of mice injected with the NCI-H226 cells (high-mesothelin-express-
ing cells) responded to SS1(dsFv)-PE38 immunotoxin. The number of lung metastases was significantly reduced from a median of 150 nodules/mouse to a median of 2 nodules/mouse (Table 1). Administration of SS1(dsFv)-PE38 also reduced lung weight (indicative of total tumor volume) in the NCI-H226 tumor-bearing animals; the lung weight of PC14PE6 tumor-bearing animals was not affected by therapy with either immunotoxin (Table 1). These results suggest that the antimesothelin immunotoxin SS1(dsFv)-PE38 produces a specific therapeutic effect against the mesothelin-expressing NCI-H226 cells growing in the lungs of nude mice.

Discussion

The membrane-bound form of mesothelin is present on the cell surface of several types of cancer cells (11, 14, 15). In some cancer patients, it is found in the circulation, but at levels that are too low to affect immunotherapy (31). These properties of mesothelin expression make it a promising targeting molecule for cancer management. The NCI-H226 human NSCLC cells express a much higher level of mesothelin than the PC14PE6 human lung adenocarcinoma cells. Under in vitro conditions, the SS1(dsFv)-PE38 antimesothelin immunotoxin was highly cytotoxic (IC_{50} = 0.008 ± 0.003 ng/ml, mean ± SE; n = 3) against the NCI-H226 cells, but not against the PC14PE6 cells (IC_{50} > 100 ng/ml). The anti-Tac(dsFv)-PE38 (anti-interleukin-2 receptor) control immunotoxin did not produce significant cytotoxicity against either cell line (IC_{50} > 100 ng/ml) under in vitro conditions. Three consecutive i.v. administrations of the SS1(dsFv)-PE38 immunotoxin eradicated significantly more lung lesions produced by NCI-H226 cells in nude mice than it did those produced by PC14PE6 cells. The specificity of the immunotoxin was confirmed by the lack of effect of the control immunotoxin anti-Tac(dsFv)-PE38 on lung cancer lesions in nude mice.

A critical therapeutic issue for the use of immunotoxins is nonspecific binding to normal tissues, which typically produces toxicity. Mesothelin is a GPI-linked glycoprotein present on the cell surface of normal mesothelial cells. GPI-linked proteins regulate a wide variety of functions in different cells.
Many of these proteins are receptors that participate in signal transduction processes; others play a role in cellular recognition and adhesion (16, 32–34). Mesothelin is a differentiation antigen, and it is expressed on the mesothelial cell lining of the lung and in the peritoneal wall of mice, an expression pattern resembling that in human tissues (16). Flat mesothelial cells regulate the movement of molecules and cells in and out of the peritoneum cavity, and mesothelin might have a role in these processes. The M, 32,000 secretory domain of mesothelin can stimulate megakaryocyte colony-forming activity of murine interleukin 3 in bone marrow cell culture of mice (12, 13, 16). Because SS1(dsFv)-PE38 does not cross-react with mouse mesothelin, the immunotoxin would be more likely to find and bind the human antigen in the animal model. Therefore, a clinical concern exists as to whether normal human mesothelin might act as a shunt for the immunotoxin molecules and hinder the efficacy of SS1(dsFv)-PE38. Mesothelin is present only on normal mesothelial cells and on the surface of several tumors, including mesothelioma and ovarian cancer (11, 14, 15). SS1(dsFv)-PE38 has been constructed against the M, 40,000 membrane-bound form of mesothelin (11) which can be detected in the blood of cancer patients at very low levels, levels too low to interfere with antibody-based therapies (4, 14). However, therapy with SS1(dsFv)-PE38 might affect normal mesothelin-producing serosal surfaces and cause inflammation or fibrosis, thus limiting the use of the immunotoxin. Future clinical trials could determine the therapeutic index for SS1(dsFv)-PE38 in specific cancer patients.

In summary, we have found that multiple i.v. administrations of recombinant SS1(dsFv)-PE38 antimesothelin immunotoxin produced by fusing the variable regions of a monoclonal antibody directed at the mesothelin in-frame with domain II and III of Pseudomonas exotoxin A can significantly decrease the establishment and growth of lung lesions produced by NCI-H226 human NSCLC cells in nude mice. The therapeutic response directly correlated with the expression level of mesothelin on these tumor cells.

### Table 1  Immunotoxin therapy against experimental lung metastases produced by NCI-H226 NSCLC cells or PC14PE6 human adenocarcinoma cells in nude mice

<table>
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<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Incidence*</th>
<th>Lung metastases</th>
<th>Lung weight (mg)</th>
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<td></td>
<td></td>
<td>No.</td>
<td>Median</td>
<td>Range</td>
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<tr>
<td>NCI-H226</td>
<td>PBS control</td>
<td>10/10</td>
<td>&gt;150</td>
<td>68 to &gt;150</td>
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<td></td>
<td>Anti-Tac(dsFv)-PE38</td>
<td>10/10</td>
<td>&gt;150</td>
<td>8 to &gt;150</td>
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<td>SS1(dsFv)-PE38</td>
<td>7/10</td>
<td>2±</td>
<td>0–39</td>
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<tr>
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<td>PBS control</td>
<td>10/10</td>
<td>61</td>
<td>18–91</td>
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<tr>
<td></td>
<td>Anti-Tac(dsFv)-PE38</td>
<td>10/10</td>
<td>51</td>
<td>10–83</td>
</tr>
<tr>
<td></td>
<td>SS1(dsFv)-PE38</td>
<td>8/10</td>
<td>65</td>
<td>7–98</td>
</tr>
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</table>

* Number of mice with tumor/number of mice injected.

*P < 0.001 (Mann-Whitney U test).

### References


Targeted Therapy against Human Lung Cancer in Nude Mice by High-Affinity Recombinant Antimesothelin Single-Chain Fv Immunotoxin 1 Supported in part by Cancer Center Support Core Grant CA16672 and Grant R35-CA42107 from the National Cancer Institute, NIH.

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