

Mesothelin Expression in Human Lung Cancer

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Abstract Purpose: To investigate mesothelin as a new target for immunotherapy in lung cancer.

Experimental Design: Mesothelin mRNA and protein expression were assessed by reverse transcription-PCR, immunoblotting, and immunohistochemistry in human lung cancer specimens. Expression was also characterized in human lung cancer cell lines by flow cytometry and immunoblotting. The SS1P immunotoxin specific for mesothelin was assessed for its cytotoxic activity against lung cancer cells.

Results: We found that mesothelin mRNA was expressed in 83% of lung adenocarcinomas (10 of 12 patients). The mesothelin precursor protein was detected in 82% of lung adenocarcinoma (9 of 11 patients), and its mature form was detected in 55% (6 of 11 patients). Immunohistochemistry showed strong and diffuse mesothelin staining in human lung adenocarcinomas and weak or modest staining in squamous cell carcinomas. We detected mesothelin mRNA in 78% of lung cancer cell lines (7 of 9) of the NCI-60 cell line panel. Mesothelin mRNA and proteins were expressed at a high level in non-small cell lung cancer lines EKVX, NCI-H460, NCI-H322M, and NCI-H522. Flow cytometric analysis showed high surface expression of mesothelin in NCI-H322M and EKVX cell lines. Immunotoxin SS1P showed high cytotoxic activity on NCI-H322M and EKVX cells with IC₅₀ values ranging from 2 to 5 ng/mL.

Conclusions: Mesothelin is expressed on the surface of most lung adenocarcinoma cells. Immunotoxin SS1P is cytotoxic against mesothelin-expressing lung cancer cell lines and merits evaluation as a new therapeutic agent in treating non-small cell lung cancer.

Lung cancer remains the most common fatal malignancy in the world. It causes nearly 170,000 cancer deaths in the United States each year, accounting for nearly 25% of all deaths from cancer (1). Lung cancers are divided into small cell lung cancers (~20%) and non-small cell lung cancers (NSCLC; ~80%); the latter are further subdivided into adenocarcinomas, squamous, and large cell cancers. Chemotherapy has helped improve the outcomes in lung cancer patients, but only 15% of patients who are diagnosed today with lung cancer will survive for 5 years. There is an urgent need to develop new drugs with different mechanisms of action. Immunotherapy represents one new approach, but it remains a challenge in part because of the lack of good target proteins. The identification of new therapeutic targets in lung cancer is of paramount importance.

Mesothelin is a glycosyl-phosphatidylinositol-anchored glycoprotein present on the cell surface of various human solid tumors. Mesothelin was first identified by the monoclonal antibody (mAb) K1 (2). The *mesothelin* (*MSLN*) gene encodes a 71-kDa precursor protein that is processed to a 40-kDa glycosyl-phosphatidylinositol-anchored protein, the mature portion to which mAb K1 binds, termed mesothelin (3), and a NH₂-terminal 31-kDa fragment called megakaryocyte-potentiating factor (4, 5) that is released from the cell. Mesothelin is a tumor differentiation antigen present at low levels on a restricted set of normal adult tissues, such as mesothelium, but aberrantly overexpressed in mesotheliomas, ovarian, and pancreatic cancers (6).

The biological functions of mesothelin remain elusive. It was originally suggested that mesothelin might have a role in cell adhesion (3). A recent study showed that mesothelin binds to MUC16/CA125, and that this interaction mediates cell adhesion (7, 8), suggesting that there may be an important role for MUC16/CA125 and mesothelin in the metastatic spread of ovarian cancer. There is evidence that in mouse mammary epithelial cells, activation of the Wnt signaling pathway can lead to an increase in mesothelin expression (9). Interestingly, ovarian and pancreatic cancers with constitutive activation of the Wnt signaling pathway have high mesothelin expression.

Mesothelin has been evaluated as a diagnostic marker for ovarian cancer and mesothelioma. Elevation of serum mesothelin in ovarian cancer and mesothelioma has been found by several groups (10–15). Serum mesothelin was also combined with other existing biomarkers, such as MUC16/CA125, to aid in the diagnosis of ovarian carcinoma (12). The exact form of mesothelin, which circulates in human plasma, was not known

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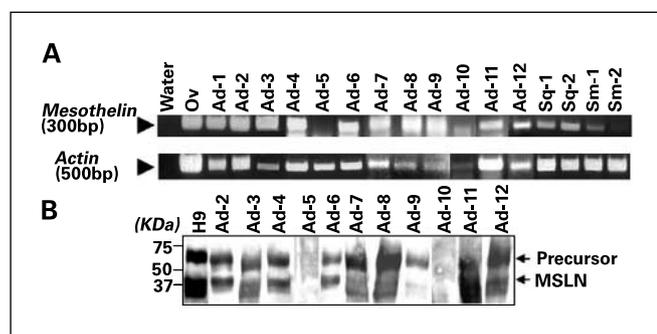


Fig. 1. Mesothelin mRNA and protein expression in frozen tissue specimens of lung cancer patients. *A*, reverse transcription-PCR detection of mesothelin mRNA in frozen tissue specimens. Water, a template-free negative control; Ov, Ovar-3 cells as a positive control; Ad, lung adenocarcinoma tissues; Sq, squamous cell carcinoma; Sm, small cell lung cancer. *B*, Western blot detection of mesothelin proteins in frozen tissue specimens. MSLN, mesothelin. H9 is the A431.MSLN⁺ (H9) stable transfected cell line used as a positive control (18).

until recently. There is now convincing evidence that a shed form of membrane-bound mesothelin is the predominant protein present in patient serum, and that serum mesothelin in ascites from a patient with ovarian carcinoma contains the sequence of the extracellular domain of membrane-bound mesothelin (16) rather than a soluble variant with no glycosylphosphatidylinositol anchorage (10). We recently provided direct evidence showing that such shedding does occur (17).

We have also found that antibodies specific for mesothelin are elevated in the sera of patients with mesothelioma and epithelial ovarian cancer, and the elevation is associated with high expression of mesothelin in tumors (18). The antibody response to mesothelin-expressing ovarian carcinoma cells may result in a reduction of tumor load and contribute to prolonged survival (19).

Mesothelin has also been suggested as a therapeutic target in mesothelioma, ovarian, and pancreatic cancers. An anti-mesothelin recombinant immunotoxin, SS1(dsFv)PE38 or SS1P, that is composed of the Fv portion of antibody SS1 and a truncated form of *Pseudomonas exotoxin* has been evaluated in phase I studies (6). In a phase I clinical trial of patients with pancreatic cancer who were vaccinated with irradiated pancreatic tumor cell lines, a strong dose-dependent T-cell antitumor immunity against mesothelin-expressing tumors was found (20). In another T-cell immunotherapy study, specific T-cell epitopes derived from mesothelin were shown to activate human T cells to efficiently lyse human tumors expressing mesothelin (21).

Mesothelin has not yet been intensively investigated in lung cancer. Two groups (22–24) have reported mesothelin expression in lung cancer by immunohistochemistry using 5B2, an anti-mesothelin antibody that was generated by immunizing mice with a recombinant protein corresponding to 100 amino acids at the NH₂ terminus of membrane-bound mesothelin (6). Miettinen and Sarlomo-Rikala (24) found that mesothelin was present in more than half (53%) of lung adenocarcinomas and a minority (13%) of large cell carcinomas but was absent in small cell carcinomas. Ordonez (22, 23) also found that about 40% of lung adenocarcinomas showed mesothelin expression, and in some of these cases, the staining was strong. These findings are in contrast to early studies using the K1 antibody, which did not detect mesothelin in 23 lung adenocarcinomas (25). It is likely that mesothelin was not

detected by mAb K1 in lung cancers because of the low affinity of the K1 antibody (6). We have recently developed two new mAbs to mesothelin, which have better binding properties than K1 (26). Here, we investigate mesothelin as a new therapeutic target for the immunotherapy of human lung cancer.

Materials and Methods

Tissues and cell lines. Frozen and fixed lung tumor samples were acquired from the Cooperative Human Tissue Network (Charlottesville, VA), which is funded by the National Cancer Institute. Nine human lung cancer cell lines in the NCI-60 cell line panel were obtained from the National Cancer Institute Anticancer Drug Screen Program. They are A549 (adenocarcinoma), Hop-62 (adenocarcinoma), Hop-92 (large cell), EKVX (adenocarcinoma), NCI-H226 (mesothelioma), NCI-H322M (adenocarcinoma), NCI-H460 (large cell), NCI-H522 (adenocarcinoma), and NCI-H23 (adenocarcinoma).

Reverse transcription-PCR analysis. Primers used in this study are Meso64 (mesothelin, sense), 5'-CGAGATAGACGAGAGCCT-3'; and Meso65 (mesothelin, antisense). Total RNAs from different cancer cell lines and cancer tissues were isolated using TRIzol reagents (Invitrogen, Carlsbad, CA). First-strand cDNA was prepared from the isolated RNA using a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). PCR was done on cDNA using the Tgo DNA polymerase with 3'-5' exonuclease proofreading activity (Roche Molecular Biochemicals, Indianapolis, IN). The PCR conditions used are initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. The expected size of the PCR products of mesothelin is shown in Fig. 1.

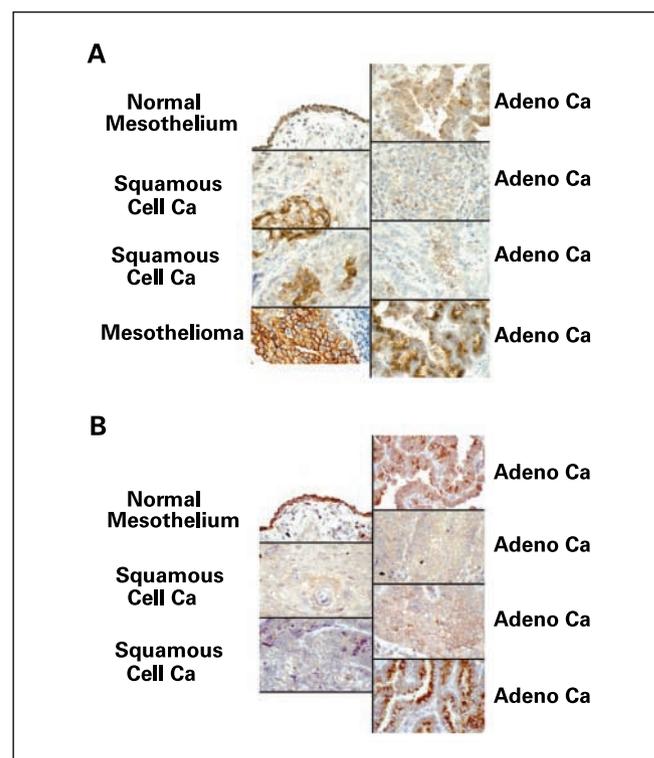


Fig. 2. Summary of immunoreactivity patterns in cancer patient samples. Immunohistochemical evaluation using two different mAbs, 5B2 (*A*) and MB (*B*), shows strong and diffuse expression of mesothelin in two of four representative lung adenocarcinoma (*Adeno*) samples. Ca, carcinoma.

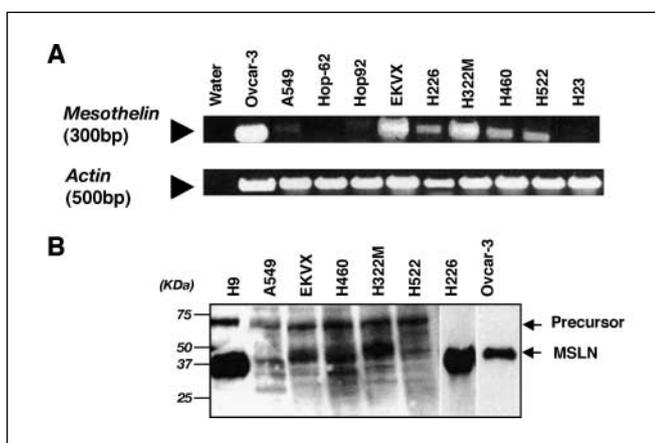


Fig. 3. Mesothelin mRNA and protein expression in the NCI-60 lung cancer cell lines. *A*, reverse transcription-PCR detection of mesothelin mRNA. Water, a template-free negative control for PCR contamination; Ovcar-3, a positive control. *B*, Western blot detection of mesothelin proteins. H9 is the A431.MSLN⁺ (H9) stable transfected cell line used as a positive control (18).

Immunohistochemistry. Tissue sections obtained from archival paraffin-embedded tumor blocks from the original biopsies of patient or surgical resection were evaluated for mesothelin expression using anti-mesothelin mAbs MB and 5B2 with peroxidase immunohistochemistry and with citraconic anhydride antigen retrieval (27).

Measurement of mesothelin expression by flow cytometry. Flow cytometric analysis was done by following an established protocol (18). Briefly, 5×10^5 cells were incubated with 1 $\mu\text{g}/\text{mL}$ of mAb (MN or MB) in 500 μL of PBS containing 5% bovine serum albumin and 0.1% sodium azide. After incubation for 1 h at 4°C, the cells were washed once with the same buffer and incubated with 1:200 dilution of phycoerythrin-labeled goat F(ab')₂ anti-mouse IgG (Invitrogen) for 1 h. After washing twice, the cells were suspended in 0.5 mL of PBS, and the fluorescence associated with the live cells was measured using a FACSCalibur flow cytometer (BD Biosciences, Palo Alto, CA). To quantitatively measure the mesothelin sites on the cell surface, the mean fluorescence intensity of cells was converted to the molecular number of fluorochromes per cell using QuantiBrite PE (BD Biosciences) calibration beads.

Western blot analysis. Immunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8), 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 $\mu\text{g}/\text{mL}$ pepstatin] containing 2% SDS was used to solubilize cells on the dish or the cell pellet in the tube. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the manufacturer's protocol. Equivalent amounts (40 μg per lane) of whole-cell lysates were separated by a 4% to 20% Tris-glycine SDS-PAGE and transferred onto nitrocellulose filters. Western blots were done using a standard method. Each blot was treated with 1 $\mu\text{g}/\text{mL}$ anti-mesothelin mAb MN. Primary antibodies were detected by secondary goat anti-mouse antibodies conjugated with horseradish peroxidase. Signals were visualized by the Enhanced Chemiluminescence kit (Amersham Biosciences).

Cytotoxicity assays. The cytotoxicity of immunotoxin SS1P was determined on NSCLC cell lines by a WST cell death assay as described previously (28).

Results and Discussion

Mesothelin expression in human lung cancers. To determine how frequently mesothelin is expressed in human lung cancer, we characterized the mRNA and protein expression of

mesothelin in lung cancer samples. We used reverse transcription-PCR to examine if mesothelin mRNA was expressed in 16 lung cancer patient specimens. The reverse transcription-PCR data in Fig. 1A show that mesothelin is expressed in 10 of 12 (83%) lung adenocarcinomas, 2 of 2 squamous cell carcinomas, and 1 of 2 small cell lung cancers. In Fig. 2B, we did Western blot experiments on 11 lung adenocarcinomas. The mesothelin precursor protein with a molecular weight of 71 kDa was detected in 9 of 11 (82%) of adenocarcinomas, and its mature form (molecular weight, 40 kDa) was detected in 55% (6 of 11; Fig. 1B). The reverse transcription-PCR and immunoblotting results show that mesothelin is expressed in most lung adenocarcinoma specimens.

We also examined mesothelin protein expression in human lung cancer specimens by immunohistochemistry. Two of four lung adenocarcinoma tissue samples had strong mesothelin immunoreactivity using two different mAbs: 5B2 (Fig. 2A) and MB (Fig. 2B). With both mAbs, the immunoreactivity was mainly distributed in the plasma membrane and the Golgi region of cells, with some signal present diffusely in the cytoplasm. Lower mesothelin immunoreactivity was observed in the other two adenocarcinomas. Some of the cytoplasmic mesothelin signals observed in lung adenocarcinoma specimens by immunohistochemistry may be due to the unprocessed 71-kDa precursor form of mesothelin detected by immunoblotting (Fig. 1B). In both squamous cell carcinomas, the immunoreactivity was greatly diminished. As previously reported in mesothelioma, there was a strong signal at the cell membrane (2). Mesothelin immunoreactivity was also seen on the membrane of normal mesothelial cells.

Characterization of mesothelin expression in lung cancer cell lines. To further characterize the expression of mesothelin in human lung cancer cells, we examined all nine lung cancer cell lines from the NCI-60 cell line panel (29): eight NSCLC lines and one mesothelioma line (NCI-H226). Among the NSCLC lines, there are six adenocarcinoma lines (A549, Hop-62, EK VX, NCI-H322M, NCI-H522, and NCI-H23) and two large cell lines (Hop-92 and NCI-H460). As shown in Fig. 3A, mesothelin mRNA was expressed in 7 of 9 (78%) lung cancer cell lines:

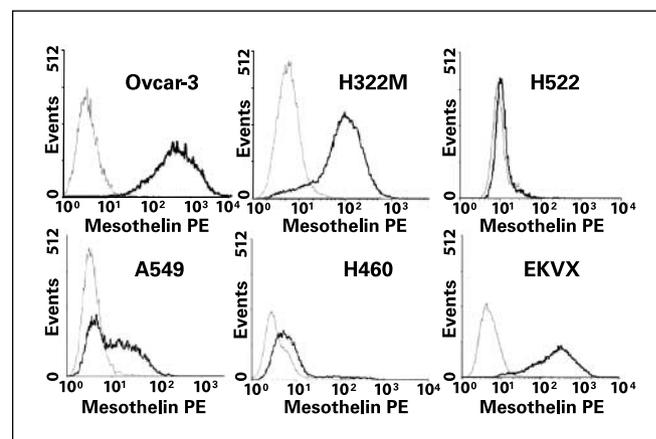


Fig. 4. Flow cytometric analysis of mesothelin protein expression in NCI-60 lung cancer cell lines. NCI-H322M (H322M), NCI-H522 (H522), A549, NCI-H460 (H460), and EK VX cells were probed with a mesothelin-specific mAb (solid dark line) or an irrelevant isotype mAb control (gray line). Ovcar-3 was used as a positive control.

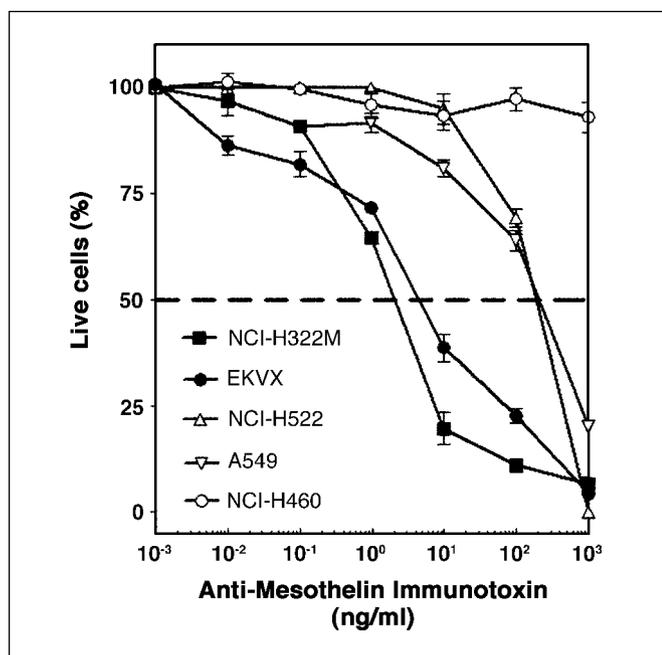


Fig. 5. Inhibition of cell viability on NCI-60 lung cancer cells by immunotoxins. Cancer cells (10,000 per well) incubated with various concentrations of anti-mesothelin immunotoxins (SS1P) for 72 h. Cell viability was determined by a WST assay as described in Materials and Methods. Points, means of triplicate determinations; bars, SD. The dashed line indicates 50% inhibition of cell viability, which is halfway between the level of viability in the absence of toxin and that in the presence of 10 μ g/mL of cycloheximide.

A549, Hop-92, EKVX, NCI-H226, NCI-H322M, NCI-H460, and NCI-H522. Both mesothelin mRNA (Fig. 3A) and proteins (Fig. 3B) were expressed at a high level in EKVX, NCI-H460, NCI-H322M, NCI-H522, and NCI-H226 cells. As shown in Fig. 3B, there are two major bands representing the 71-kDa precursor and 40-kDa mature forms of mesothelin in all the NSCLC lines. Some minor and low-molecular weight bands were detected probably due to the degradation of mesothelin. In the mesothelioma (NCI-H226) and ovarian cancer (Ovar-3) lines, the 40-kDa mature form is the dominant form present.

To determine how much mesothelin is present on the surface of NSCLC cells, we did flow cytometric analysis of A549, EKVX, NCI-H322M, NCI-H460, and NCI-H522 cells. As shown in Fig. 4, adenocarcinoma NCI-H322M and EKVX cell lines had

the highest surface expression (~100,000 sites per cell) of mesothelin proteins, and A549, NCI-H460, and NCI-H522 cell lines had modest surface expression (6,000-10,000 sites per cell) of mesothelin.

Cytotoxicity of mesothelin-specific immunotoxin SS1P on human lung cancer cell lines. To determine if mesothelin could function as a new therapeutic target in lung cancer, we tested an anti-mesothelin recombinant immunotoxin, SS1(dsFv)PE38 or SS1P, that is composed of the Fv portion of antibody SS1 and a truncated form of *Pseudomonas* exotoxin. SS1P has been evaluated in phase I studies for mesothelioma and ovarian cancer patients (6). The cytotoxicity of immunotoxin SS1P was determined on five NSCLC cell lines (A549, EKVX, NCI-H322M, NCI-H460, and NCI-H522) by a WST cell death assay (Fig. 5). In NCI-H322M and EKVX, the two adenocarcinoma cell lines with the highest mesothelin expression on the cell surface (Fig. 4), SS1P was very active with IC₅₀ values ranging from 2 ng/mL (NCI-H322M) to 5 ng/mL (EKVX). In the A549 and NCI-H522 cell lines with low mesothelin expression, lower but significant cytotoxic activity (IC₅₀ = 200 ng/mL) was observed.

In the present study, we found that mesothelin mRNA and protein are present in a substantial number of lung adenocarcinomas. Expression was confirmed using cell lines where mesothelin mRNA was detected in seven of nine lines of the NCI-60 panel. Furthermore, flow cytometric analysis showed high surface expression of mesothelin in the NCI-H322M and EKVX lung adenocarcinoma cell lines.

One unusual feature of the lung cancer samples is that there is more intracellular mesothelin reactivity than in mesotheliomas, ovarian cancers, or normal mesothelial cells. In addition, there seems to be substantial amounts of the high-molecular weight mesothelin precursor present in lung cancers, possibly accounting for its intracellular location.

Two phase I clinical trials of immunotoxin SS1P were recently completed at the National Cancer Institute in mesothelioma and ovarian cancer patients. Several minor but significant antitumor responses were observed.³ Phase II studies of SS1P will begin in 2007. In the current study, we showed that immunotoxin SS1P, specific for mesothelin, has a strong cytotoxic activity on NCI-H322M and EKVX cell lines with IC₅₀ values ranging from 2 to 5 ng/mL. This work suggests that SS1P also merits evaluation for the treatment of NSCLC.

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³ R. Hassan and I. Pastan, unpublished data.

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