Circulating Mesothelin Protein and Cellular Antimesothelin Immunity in Patients with Pancreatic Cancer

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Abstract

Purpose: Mesothelin is a glycoprotein expressed on normal mesothelial cells and is overexpressed in several histologic types of tumors including pancreatic adenocarcinomas. A soluble form of mesothelin has been detected in patients with ovarian cancer and malignant mesothelioma, and has prognostic value. Mesothelin has also been considered as a target for immune-based therapies. We conducted a study on the potential clinical utility of mesothelin as a biomarker for pancreatic disease and therapeutic target pancreatic cancer.

Experimental Design: Tumor cell–bound and soluble mesothelin in patients was evaluated by immunohistochemistry and ELISA, respectively. The in vitro cellular immune response to mesothelin was evaluated by IFNγ ELISA and intracellular cytokine staining for IFNγ in CD4+ and CD8+ T cells. The level of circulating antibodies to mesothelin was measured by ELISA.

Results: All tumor tissue from patients with pancreatic adenocarcinoma expressed mesothelin (n = 10). Circulating mesothelin protein was detected in patients with pancreatic adenocarcinoma (73 of 74 patients) and benign pancreatic disease (5 of 5) but not in healthy individuals. Mesothelin-specific CD4+ and CD8+ T cells were generated from peripheral blood lymphocytes of patients with pancreatic cancer in 50% of patients compared with only 20% of healthy individuals. Antibodies reactive to mesothelin were detected in <3% of either patients or healthy individuals.

Conclusions: Circulating mesothelin is a useful biomarker for pancreatic disease. Furthermore, mesothelin-specific T cells can be induced in patients with pancreatic cancer. This suggests that mesothelin is a potential target for immune-based intervention strategies in pancreatic cancer. (Clin Cancer Res 2009;15(21):OF1–8)

Pancreatic adenocarcinoma is the fourth leading cause of cancer mortality in the United States (1). The vast majority of patients present with incurable metastatic disease, and there remains a lack of both tests for early diagnosis as well as effective therapies. Recent work has suggested that the immune response plays an important part in the pathogenesis of this malignancy (2–4). In particular, the presence of tumor-infiltrating CD4+ and CD8+ T cells as well as dendritic cells has been associated with improved prognosis (5). Furthermore, it has been shown that this immune response may be mediated in part by tumor-associated antigen(s) (refs. 2, 4, 6). One such candidate antigen is mesothelin (7, 8). The mesothelin gene encodes a 71-kDa precursor that is processed into the 40-kDa GPI-anchored protein, mesothelin, and a 31-kDa fragment called megakaryocyte potentiating factor (7,10). Although its function remains unclear, mesothelin is constitutively expressed by mesothelial cells, but has been found to be overexpressed in pancreatic adenocarcinoma (11) and in a number of other solid tumors, such as lung (12) and ovarian (7,13–15) cancer. Importantly, when overexpressed in patients with ovarian cancer or mesothelioma, circulating mesothelin has been detected (14–20). Due
Novel diagnostic biomarkers and therapeutic agents are much needed for patients with pancreatic cancer. Mesothelin is a differentiation antigen present on normal mesothelial cells. It is also highly expressed in pancreatic cancer. Circulating mesothelin is readily detectable in patients with pancreatic disease using a specific ELISA and thus serves as a useful biomarker for malignant and benign pancreatic tumors. The differential expression of mesothelin in normal versus cancer tissues makes mesothelin an attractive candidate for targeted therapies such as mesothelin-based immune therapies. Understanding the immunologic mechanisms for antimesothelin immunity in pancreatic cancer allows for the rational design of therapeutic strategies to improve clinical outcomes for patients afflicted with this deadly disease.

**Materials and Methods**

**Patient characteristics.** Peripheral blood was collected from 81 patients with pancreatic disease of which 74 (91%) were diagnosed with pancreatic adenocarcinoma, 2 patients with neuroendocrine tumors of pancreatic cancer and 5 patients with benign pancreatic disease (6%). The median age of the patients was 69 y (range, 44–97 y) with a male to female ratio of 54:27. Clinical staging for patients with pancreatic adenocarcinoma was as follows: stage IV (25%), stage III (17%), stage IIb (42%), stage IIA (12%), and stage IB (4%). Five patients with benign pancreatic disease were included: two males and three females ranging in age from 67 to 71 y. Four of five were diagnosed with intraductal papillary mucinous neoplasm (IPMN) and one patient with mucinous cystic neoplasm. All specimens were collected under research protocols approved by the Washington University School of Medicine Human Studies Committee after informed consent was obtained.

**Peripheral blood samples.** Peripheral blood samples were obtained from patients with pancreatic disease and normal healthy volunteers in part through the Siteman Cancer Center Hereditary Cancer Core. EDTA (plasma) tubes and serum tubes (BD Vacutainer tubes, BD Biosciences) were obtained from each patient. EDTA tubes were centrifuged at 2,000 × g for 10 min at room temperature. The resultant supernatant was spun again at 2,500 × g for 15 min, and plasma was collected. Serum tubes were allowed to clot for 30 min at room temperature, then spun at 300 × g for 5 min. Samples were kept at -80°C until use.

**Isolation of peripheral blood mononuclear cells.** Peripheral blood mononuclear cells (PBMC) were isolated as previously described (29). Briefly, heparinized blood underwent Ficoll density centrifugation, and the buffy coat containing PBMC was harvested and washed twice in PBS. PBMC were subsequently used in functional studies or cryopreserved.

**Cell culture and intracellular cytokine analysis.** PBMC from each patient were diluted to 2 to 3 × 10⁶ cells per mL. Cells were placed in 1 mL AIM-V medium (Invitrogen) supplemented with 2.5% Human AB serum (Sigma), 1% l-glutamine, and 1% penicillin/streptomycin (both Mediatech). Cultures were left un pulsed or were pulsed with purified native mesothelin protein (20 μg/mL) or anti-CD3 antibody (OKT3, 1 μg/mL). After 5 to 7 d, culture medium was harvested, cells were spun down, and supernatant was frozen at -20°C. Alternatively, 1-wk-old mesothelin-stimulated cultures were harvested, were spun down, and cells were kept for 24 h in cytokine-free medium. The next day, cells were restimulated at a 1:1 ratio with autologous, irradiated PBMC either un pulsed or pulsed with mesothelin protein, and 24 h later, supernatant was harvested and tested for IFNγ. In follow-up studies, 1-wk-old cultures were restimulated for a second week. These cultures received interleukin-2 (II-2) at 50 IU/mL every 3 d. After an additional 5 to 7 d, the cells were washed in medium without II-2 and placed into culture medium without II-2 for 24 h. Subsequently, the cells were restimulated for 24 h at a 1:1 ratio with autologous, irradiated PBMC either unpulsed or pulsed with mesothelin protein. Brefeldin A (eBioscience) was added at 3 μg/mL final concentration for the last 5 h of restimulation before harvesting for antibody staining. Cells were generally blocked with mouse serum before incubating with specific staining antibodies. Intracellular staining for IFNγ was done according to the manufacturer's (eBioscience) reagents and instructions; matched iso types were used to determine positive and negative cell populations. Briefly, cell suspensions were first stained for CD4 and CD8 (BD Pharmingen), fixed at room temperature for 20 min, then permeabilized in eBioscience Permeabilisation buffer before staining for IFNγ (eBioscience). Cells were analyzed using a FACS Calibur flow cytometer and FlowJo software (TreeStar).

**IFNγ ELISA.** The amounts of IFNγ in the culture supernatants were measured by ELISA using the Human IFNγ Cytoset assay (Biosource) according to the manufacturer's instructions. A standard curve was included in each assay. The minimum detection level of IFNγ is 7 pg/mL.

**Mesothelin ELISA.** Soluble mesothelin was measured by a validated double determinant sandwich ELISA (18). Detection was measured by absorbance in an ELISA reader (SpectraCount Microplate Photometer, Packard). Monoclonal antibodies (mAbs) to two spatially different epitopes, mAbs OV569 and 4H3, were used to study serum and plasma samples. Mesothelin levels were determined as absorbance according to absorbance measurement by a microplate reader at 450 nm (18) and are called absorption units (AU). As reported earlier for this assay (15, 17–19), a sample was considered positive if AU of ≥0.20 at 1:40 dilution, which is 3 SDs above the mean absorbance measurement at 450 nm for over 100 healthy controls tested previously (19). Samples were diluted in PBS containing 3% bovine serum albumin (BSA). All assays included either PBS/3% BSA or a healthy volunteer sample as a negative control, and serum from a patient with ovarian carcinoma was used as positive control on the same ELISA plate (19).

**Antimesothelin antibody ELISA.** Peripheral blood was evaluated for the presence of antimesothelin IgG antibodies using a recently developed ELISA (19). In this assay, plates are coated with native purified human mesothelin at 10 μg/mL. After incubation of serum samples, plates are incubated with horseradish peroxidase-conjugated mouse anti-human IgG antibody. Samples were tested at 1:20 and 1:80 dilutions in PBS/3% BSA and absorbance was determined at 450 nm. AU of ≥0.5 was used as cutoff for a positive sample at 1:20 dilution of samples. PBS/3% BSA was added to some wells as negative control, and serum from a patient with ovarian cancer was used as positive control (19).

**Mesothelin protein.** Two sources of mesothelin protein were used in this study. Initial studies were done with a recombinant human mesothelin-human IgG fusion protein (18, 20) with an estimated purity of ~60% as determined by gel electrophoresis and Coomassie staining. The majority of the studies were done with native human mesothelin.
purified from either urine of patients with ovarian or pancreatic cancer, or culture medium from a mesothelioma cell line (19). The identity of the purified native mesothelin was earlier confirmed by mass spectrometry (19). The protein solution was further characterized by gel electrophoresis followed by staining of the gel with SYPRO Ruby, and Western blot using the anti-mesothelin (K1) mAb (Santa Cruz Biotechnology, Inc.) followed by peroxidase-conjugated goat anti-mouse IgG (Fig. 1).

Immunohistochemistry. Paraffin-embedded tissue sections of archival human pancreatic ductal adenocarcinoma were evaluated for expression of mesothelin. Detection of mesothelin by immunohistochemistry was done by using the anti-human mesothelin–specific mAb, 5B2 (Novocastra Laboratories, Ltd) following the manufacturer’s instructions. This antibody has been well characterized and been used in immunohistochemistry to assess its diagnostic potential (30). Representative formalin-fixed, paraffin-embedded tissue blocks containing invasive pancreatic ductal adenocarcinoma and normal tissue was chosen for labeling. A positive reaction was defined as discrete localization of the brown chromagen on the cell surface. Detection of immunolabeling was done by an independent observer (Dr. E.M. Brunt, a pathologist at our institution). All cases demonstrating >25% labeling were categorized as “positive” and scored on a scale from 1 to 3 with 3 being the highest.

Statistical analysis. The circulating mesothelin levels in patients with benign, primary, and metastatic pancreatic cancer were compared with those in healthy volunteers using a nonparametric Mann-Whitney test. Analysis of ELISA results for IFNγ was done by a Student’s t test. Intracellular IFNγ levels in patients versus healthy controls were compared by the nonparametric Mann-Whitney test. In all analyses, a P value of <0.05 was considered significant.

Results

Pancreatic adenocarcinomas express mesothelin. Ten surgically resected specimens of human pancreatic adenocarcinoma were stained for mesothelin (Fig. 2). All samples were positive, with immunoreactivity distributed along the plasma cell membrane. The luminal surface of tumor glands but not of normal glands stained positive for mesothelin. This staining pattern is consistent with previous reports of mesothelin expression in pancreatic and other tumors (11, 30–32).

Elevated circulating mesothelin levels in patients with pancreatic disease. As overexpression of mesothelin in other types of cancer results in soluble mesothelin detectable in peripheral blood, we evaluated patients with pancreatic disease for circulating mesothelin. To test whether the ELISA for circulating mesothelin yields similar results for serum and plasma samples, a side-by-side comparison was done using serum and plasma samples collected from the same patients. No significant difference was observed in soluble mesothelin levels between plasma and serum samples from the same patients tested at the same dilution (Fig. 3A). Using this ELISA, 73 of 74 (99%) patients with pancreatic adenocarcinoma had elevated circulating levels of mesothelin compared with none of 5 healthy controls (Fig. 3B). In addition, all 5 patients with biopsy-proven benign pancreatic disease had elevated levels of circulating mesothelin. The median AU in benign disease patients (0.405) was not significantly different from that in patients with either resectable (0.409) or unresectable/metastatic pancreatic cancer (0.497). There was no significant difference in mean AU comparing patients with resectable or unresectable disease. However,

Fig. 1. Analysis of purified native mesothelin by gel electrophoresis and Western blot. A, soluble mesothelin purified from culture supernatant of a mesothelioma cell line or urine from ovarian and pancreatic cancer patients (19) was run on a 4% to 12% SDS gel (lane 2) and stained by SYPRO-Ruby. Lane 1 was loaded with molecular weight markers. B, two duplicate lanes were used for further development by Western blot using the anti-human mesothelin mAb, K1, in lane 1 or serum from a patient with pancreatic adenocarcinoma negative for antimesothelin antibody (lane 2).

Fig. 2. Human pancreatic adenocarcinoma expresses mesothelin. Representative micrographs at low (A) and high (B) power of human pancreatic adenocarcinoma stained for mesothelin. Tissue sections were stained with the anti-human mesothelin mAb, 5B2, and counterstained by H&E. Nontumor pancreatic glands are nonreactive with the mesothelin antibody (arrow, A). Only tumor glands (arrow, B) stain positive for mesothelin. The malignant tumor epithelium is 3+ in all the tumor glands.
the median AU in healthy controls was 0.151 and was significantly lower than the mean AU for the patient populations ($P < 0.001$).

**Mesothelin-specific cellular immune responses.** The increased levels of mesothelin protein present in the circulation of patients with pancreatic cancer could potentially have an impact on mesothelin-specific immunity. We therefore performed a series of experiments evaluating the presence of mesothelin-specific T cells in PBMC of pancreatic cancer patients. First, mesothelin-pulsed PBMC were evaluated for production of IFN-γ after 5 to 7 days of culture. In three of six patients, the mesothelin-pulsed PBMC produced markedly increased levels of IFN-γ compared with PBMC alone (Fig. 4A, Pts38, 42, and 43). All six patients produced high levels of IFN-γ after anti-CD3 stimulation (data not shown). To further characterize this response, mesothelin-stimulated T cells were rested overnight and restimulated the next day with irradiated, autologous PBMC or PBMC pulsed with either mesothelin or the breast cancer-associated protein, mammaglobin. After 24 hours, supernatant from both cultures were evaluated for the presence of IFN-γ. A markedly increased production of IFN-γ by the mesothelin-stimulated T cells was observed compared with control PBMC- or mammaglobin-pulsed PBMC-stimulated T cells in three of four patients with pancreatic cancer by ELISA (Fig. 4B).

**Mesothelin-reactive CD8 and CD4 T cells in pancreatic cancer patients.** To more definitively show the presence of mesothelin-specific T cells, twice stimulated T-cell cultures were rested for 24 hours in culture medium without IL-2. T cells were subsequently restimulated with either irradiated autologous PBMC or PBMC pulsed with mesothelin protein, and analyzed for intracellular IFN-γ. These studies were done with PBMC from both patients with pancreatic cancer and healthy controls. Cells were stained for CD4, CD8, and intracellular IFN-γ, then flow cytometry was done (Fig. 5A). An increased percentage of IFN-γ-positive cells were detectable in four of seven patients after mesothelin stimulation compared with control stimulation (Fig. 5B and C). The marked increase in IFN-γ-positive cells was detected in both the CD4 and CD8 subset. In contrast, in parallel cultures of age-matched,
cancer-free controls, only minor responses were observed in the CD4 subset of two of four cultures (Fig. 5B) with no IFNγ+ CD8+ T cells detectable in any of the four controls (Fig. 5C). Similar studies in an additional six healthy volunteers confirmed the observations in the age-matched controls (data not shown). As a group, the percentages of IFNγ+ cells in both the CD4 and CD8 subsets of patients were significantly higher than in healthy controls (P < 0.05). Identical studies performed in four patients
with benign pancreatic disease, in particular patients with IPMNs, indicated the presence of mesothelin-specific CD4 and CD8 T cells in two patients (Fig. 5D, Pts 46 and 47). Of those two, patient 46 was diagnosed with IPMN containing small foci of invasive cancer.

**Pancreatic cancer patients do not develop increased IgG antibodies to mesothelin.** Using an ELISA specific for antimesothelin IgG, we determined the presence of specific IgG antibodies to mesothelin present in the serum or plasma of patients with pancreatic adenocarcinoma. A positive reaction was defined as an AU of ≥0.5 at a 1:20 diluted sample (19). Antibodies to mesothelin were only detected in 1 of 56 patients (AU = 0.538) that included patients with resectable and unresectable/metastatic pancreatic cancers patients on standard (chemo) therapy, and 1 of 35 healthy donors (AU = 0.562), 14 of which were age-matched controls (Fig. 6).

**Discussion**

In this study, we sought to explore the clinical utility of mesothelin as a biomarker and therapeutic target in patients with pancreatic adenocarcinoma. First, we confirmed that mesothelin is expressed in virtually all tumor specimens. Second, we showed that patients with pancreatic disease have elevated circulating levels of mesothelin compared with healthy controls, and lastly, we show that mesothelin-specific CD4 and CD8 T cells can be induced from patient-derived PBMC.

Mesothelin is a cell surface protein expressed in many epithelial cancers (30, 33, 34). Its unique expression pattern makes it a potential target for immune intervention. Earlier studies in ovarian cancer and mesothelioma have shown that the extracellular, GPI-linked domain of mesothelin can be cleaved off of the cell surface, resulting in a soluble mesothelin protein of approximately 40 to 45 kDa (9, 10, 33). Having confirmed previous findings that mesothelin is overexpressed in pancreatic adenocarcinoma, we sought to determine first whether circulating mesothelin could be detected in patients with pancreatic disease, second, whether soluble levels of mesothelin are elevated in pancreatic cancer patients compared with healthy controls, and, third, whether this is associated with increased antimesothelin immunity. Our data show that soluble mesothelin levels are significantly increased in patients with pancreatic disease compared with healthy controls (see Fig. 3B). Furthermore, there was no detectable evidence for high-affinity antimesothelin IgG antibodies in patients. Lastly, mesothelin-specific CD4 and CD8 T cells were readily detectable in pancreatic cancer patient-derived PBMC after *in vitro* expansion. These data suggest that (a) circulating mesothelin is a biomarker of pancreatic disease, and (b) immune tolerance to mesothelin can be overcome (e.g., by culturing the T cells in the absence of immunosuppressive mechanisms in the tumor-bearing host) and suggest that mesothelin is an attractive target for immune-based therapies.

The normal biological function of cell membrane-bound mesothelin remains elusive. Mutant mice with targeted mesothelin gene inactivation are normal, exhibiting no apparent anatomic, hematologic, or reproductive abnormalities (13). It was originally suggested that mesothelin might have a role in cell adhesion (33). Studies in ovarian cancer (35, 36) have suggested that by binding to CA125/MUC16 on the surface of tumor cells, mesothelin mediates cell adhesion, and thereby plays a role in metastasis (36). Other studies in pancreatic cancer have suggested a role for mesothelin in tumorogenesis by increasing cell proliferation, migration, and S-phase cell population (8). Thus, from a cellular biological point of view, the apparent redundant homeostatic function of mesothelin combined with growing evidence of its role in carcinogenesis strengthens its candidacy as a tumor antigen worth targeting.

Mesothelin was first described by Pastan and colleagues (32, 33) as the cell surface component of a precursor molecule that also encodes soluble megakaryocyte-potentiating factor. The GPI-anchored mesothelin is shed from tumor cells, as was first shown by Scholler et al. (18) who developed a sandwich ELISA for detection of soluble mesothelin. This ELISA has since been used in a number of studies including the studies presented here, and an assay to measure mesothelin has been commercialized as MESOMARK for studies on mesothelioma (37). To our knowledge, this is the first report on detection of soluble mesothelin in patients with pancreatic disease. The assay can be performed with either serum or plasma, as our data suggest (Fig. 3B). Elevated levels of soluble mesothelin were detected in the circulation of 73 of 74 patients tested (Fig. 3A), resembling the high frequency of overexpression of mesothelin in pancreatic cancer; in contrast, circulating mesothelin was not detected in healthy controls, which is in agreement with earlier studies on much larger control populations (38). Thus, an increased level of circulating mesothelin indicates pancreatic neoplasm (benign or malignant). Encouraged by these findings, we sought to determine the utility of soluble mesothelin as a marker of pancreatic cancer and, more specifically, as a marker of tumor burden. To that end, we first measured mesothelin levels in patients with benign as well as malignant pancreatic disease. Soluble mesothelin was detected in five of five patients with benign pancreatic neoplasms at levels not significantly different from patients with malignant pancreatic disease, indicating that measurement of circulating mesothelin does not distinguish between benign and malignant pancreatic neoplasms for which a different approach would be needed. We then attempted to correlate the levels of soluble mesothelin with time to recurrence or, in all patients, time to death. We also evaluated if the level of
soluble mesothelin decreased after surgery, and whether the difference between presurgery and postsurgery levels had prognostic value. Finally, we tested if high levels using an arbitrary cutoff either presurgery or postsurgery were predictive of clinical outcome. However, no statistically significant correlations were detected, suggesting that mesothelin does not accurately reflect the individual patient’s tumor burden. This outcome seems disease related, as the same assay and source of samples does predict clinical outcome in diseases such as ovarian cancer and mesothelioma (17, 19, 38). It could well be that the rapid recurrence of pancreatic cancer (50% of patients have recurrent disease within 1 year of surgery) suggests that very few patients are truly disease free for a period of time and may explain the lack of predictive value of soluble mesothelin in pancreatic cancer.

In addition to evaluation of the diagnostic usefulness of soluble mesothelin, we determined if increased levels of soluble mesothelin translated into increased antimesothelin immunity. However, only 1 of 56 patients tested positive for mesothelin antibody (Fig. 6). mAbs against mesothelin coupled to toxins are currently being tested in clinical trials of passive immunotherapy in pancreatic and ovarian carcinoma (39–41). It is however possible that antimesothelin antibodies themselves have therapeutic activity similar to for example trastuzumab, the anti-Her-2/neu antibody. T-cell responses to mesothelin were readily detectable in PBMC from pancreatic cancer patients after in vitro expansion with either a purified human mesothelin-Ig fusion protein or native human mesothelin. Both CD4 and CD8 T-cell populations contained mesothelin-specific T-cells with the ability to produce IFNγ upon activation (Fig. 5). Similarly, Thomas et al. (23) showed that three rounds of in vitro stimulation were required to show CD8 T-cell–mediated lysis of mesothelin-expressing tumor lines using T cells from pancreatic cancer patients undergoing immunotherapy. Interestingly, we identified mesothelin specific T-cell response in half of the patients with benign pancreatic disease. It is important to note that although IPMN is benign, it is a well recognized risk factor for the development of a future pancreatic cancer. These results suggest that an immune response to mesothelin may emerge early in cancer development.

Several T-cell epitopes encoded by human mesothelin protein have been identified in vitro (23, 25, 28). Furthermore, mesothelin-specific T cells were detected in patients with metastatic pancreatic cancer after vaccination with allogeneic granulocyte macrophage colony-stimulating factor–secreting tumor cells with or without cyclophosphamide that favorably correlated with a better clinical course (23, 25). Together with our findings here, these data confirm the immunogenicity of mesothelin and support the notion that mesothelin is an attractive target for immune-based therapies in pancreatic cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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