

Discovery of Mesothelin and Exploiting It as a Target for Immunotherapy

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Abstract

We have recently reported that an immunotoxin targeting mesothelin produced durable major tumor regressions in patients with extensive treatment-refractory mesothelioma. These unprecedented tumor responses have prompted us to review how mesothelin was discovered and the advances that led to these tumor responses. This review is not comprehensive but focuses on major developments over the past 20 years since mesothelin was first identified in our laboratory. Mesothelin is a cell-surface glycoprotein whose expression in normal human tissues is restricted to mesothelial cells. Because it is highly expressed by many solid tumors, it is an attractive immunotherapy target. Antibody-based therapies currently in clinical trials include an immunotoxin, a chimeric monoclonal antibody, and an antibody drug conjugate. In addition, a mesothelin tumor vaccine and a mesothelin- chimeric antigen receptor are being evaluated in the clinic. SS1P, an anti-mesothelin immunotoxin, was the first mesothelin-directed therapy to enter the clinic, and its use showed that mesothelin-targeted therapy was safe in patients. More importantly, our recent work has shown that SS1P in combination with pentostatin and cyclophosphamide can result in durable tumor regression in patients with advanced mesothelioma and opens up the possibility that such an approach can benefit patients with many common cancers. *Cancer Res*; 74(11); 2907–12. ©2014 AACR.

Discovery of Mesothelin

In the early 1990s, Ira Pastan and Mark Willingham (National Cancer Institute, NIH), realizing there were very few targets on the plasma membrane of solid tumors that were useful for antibody-based therapies, initiated a search for new antibodies that recognized cell-surface proteins highly expressed on cancers and not expressed on essential normal tissues so that undesirable side effects would not occur when antibodies were given to these patients. To make new monoclonal antibodies (mAb), they used standard hybridoma methodology, but to prevent mice from making antibodies to normal tissue antigens, they added a step in which mice were tolerized to normal human proteins by first immunizing them with normal liver or kidney membranes and then treating with cyclophosphamide to kill the B cells activated by this immunization. In the experiment that led to the discovery of mesothelin, they were looking for a new antibody to ovarian cancer and thus the mice were immunized with an ovarian cancer cell line (OVCAR3). After isolation of candidate mAbs, they used immunohistochemistry on frozen sections of normal tissues to exclude mAbs reacting with essential organs.

In 1992, these investigators reported on an antibody reacting with ovarian cancers named mAb K1 (1). Immunohistochemical studies performed on normal human and monkey tissues showed that the reactivity of mAb K1 was limited to the mesothelial cells of the pleura, peritoneum, and pericardium, as well as cells of the fallopian tubes and tonsils (1). The mAb was subsequently shown to react with malignant mesotheliomas, as well as squamous cell carcinomas of the esophagus and cervix (2, 3). The antibody was given the name K1, to acknowledge the contribution of Kai Chang (National Cancer Institute, NIH), the postdoctoral fellow who worked on the project.

The K1 antibody has low affinity; it reacts with frozen tissues but not as well with formalin-fixed tissues, presumably because the epitope it recognizes is destroyed by fixation. Subsequent studies using an antibody made to a peptide that reacts with fixed tissues showed that mesothelin was also present in cancers of the pancreas, lung, stomach, bile ducts, and triple-negative breast cancer (4–7). It was estimated that mesothelin is expressed in 30% of human cancers and is therefore a very important target for immunotherapy (8).

Protein Characterization and Cloning

To identify the protein reacting with mAb K1, proteins on the cell surface were labeled with ^{125}I and the cells were treated with phospholipase C to release surface proteins. The proteins released were subjected to SDS-PAGE followed by Western blotting. The antibody recognized a protein with a molecular weight of 40 kDa on both OVCAR3 and HeLa cells. The K1 mAb was then used to screen a lambda cDNA expression library made from HeLa cells. The cDNA that was isolated encoded a

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69-kDa protein, much larger than the 40-kDa protein detected on the surface of cells (9). When the cDNA was expressed in 3T3 cells, a major 40-kDa band and a minor 69-kDa band were detected indicating that the 40-kDa band was derived from a larger protein. Furthermore, analysis of the DNA sequence showed that the C terminus of the protein was characteristic of proteins, which are attached to the plasma membrane by phosphatidyl inositol. Because the protein was expressed in normal mesothelial cells, we named the gene and the protein it encoded as mesothelin.

Cell-surface mesothelin is almost exclusively of the 40-kDa-glycosylated form. The amino terminal peptide named MPF (megakaryocyte potentiating factor) is released from cells by

the action of the protease furin (Fig. 1A). MPF was initially identified as a factor produced by a pancreatic cancer cell line that had the ability in the presence of interleukin-3 to stimulate megakaryocyte differentiation in mice (10). Its function in humans is not clear at this time.

Immunotherapy Target

To determine whether mesothelin would be a useful target for antibody-based therapies, Hassan and colleagues carried out experiments in mice subcutaneously bearing grafted human tumors expressing mesothelin. In the first experiment, the K1 antibody was labeled with indium¹¹¹ and specific tumor

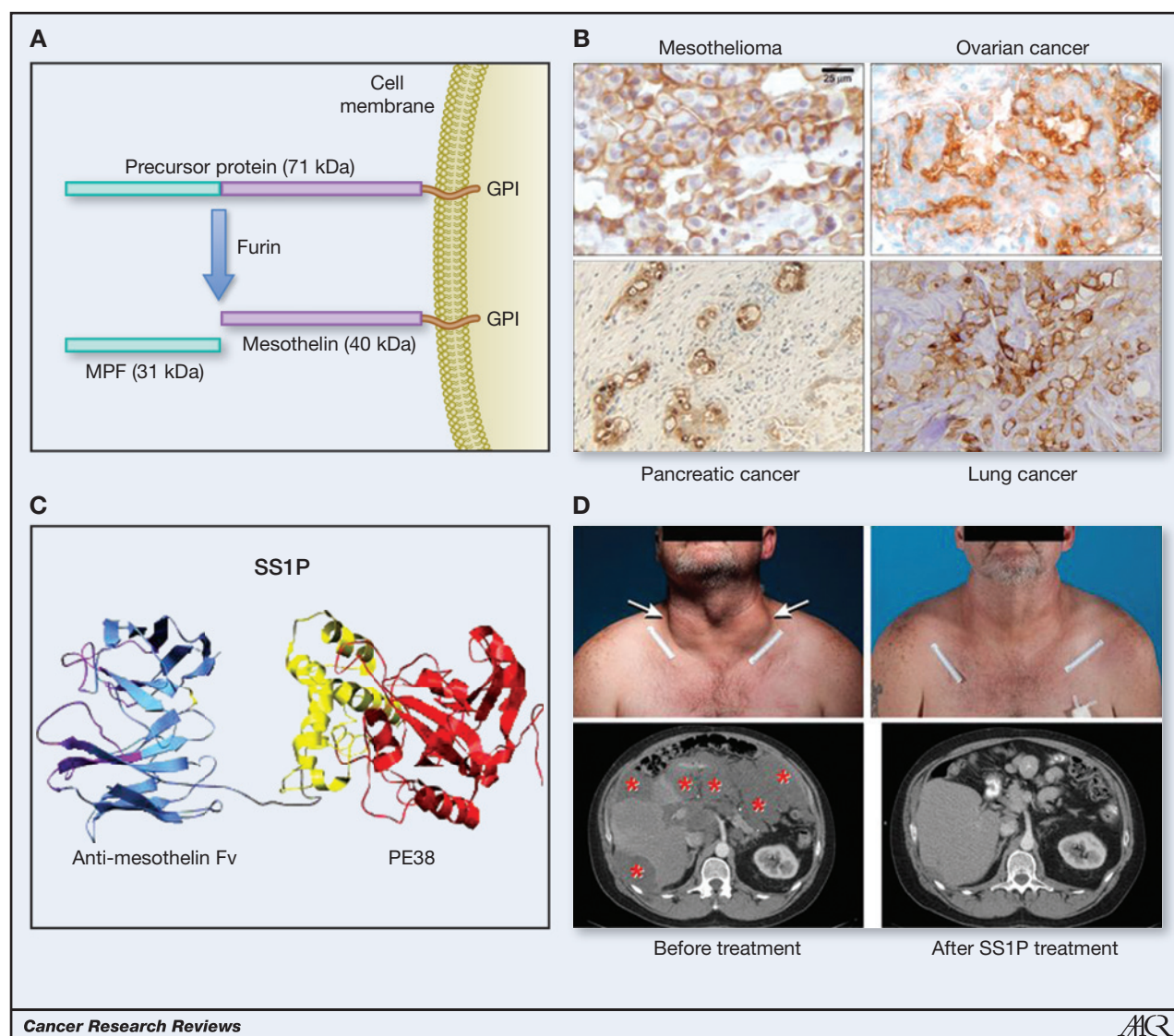


Figure 1. Processing of the 71-kDa mesothelin precursor protein to MPF and membrane-bound mesothelin by the protease furin. A, mesothelin is attached to the cell membrane by a GPI anchor. B, mesothelin expression detected in different human tumors by immunohistochemistry using an anti-mesothelin antibody. Mesothelin positivity is shown by brown staining of the tumor cells. C, schematic representation of the immunotoxin SS1P consisting of an anti-mesothelin Fv linked to the truncated *Pseudomonas* exotoxin, PE38. D, major tumor shrinkage in the neck and abdomen of a patient with metastatic peritoneal mesothelioma treated with SS1P in combination with pentostatin and cyclophosphamide. A portion of this figure (B) was originally published in an article by Hassan and Ho in the Eur J Cancer 2008;44:46–53, Elsevier publishers.

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uptake of the labeled antibody was demonstrated (11). To determine whether the antibody could be used to kill tumor cells, a fragment of *Pseudomonas* exotoxin A (PE) was attached to the antibody and the resulting immunotoxin (K1-LysPE38QQR) was shown to kill mesothelin-expressing cell lines and to cause regressions of a mesothelin-expressing tumor in mice (12). These two experiments, as well as the frequent distribution of mesothelin on cancers and the limited expression on normal tissues, provided key evidence that mesothelin is an excellent target for antibody-based therapies and encouraged the development of agents that could be used in patients.

Because the K1 antibody has a low affinity for mesothelin, a new antibody termed SS was produced by immunizing mice using a mesothelin-expressing cDNA construct. The technique of phage display was used to isolate an Fv that bound to mesothelin (13). The affinity of SS was low, but was improved to a Kd of about 1 nmol/L by mutagenizing residues in the CDR3 of the heavy chain of the Fv using a new technique called hot spot mutagenesis (14). The new Fv (SS1) was used to make a recombinant immunotoxin, termed SS1P, by fusing the Fv to a 38-kDa fragment of PE. Recombinant immunotoxins are chimeric proteins in which a tumor-specific Fv is attached to a portion of PE or other protein toxins (15). SS1P kills cells by binding to mesothelin, entering cells by receptor-mediated endocytosis, inactivating elongation factor 2, arresting protein synthesis, and inducing apoptosis.

Blood Test for Mesothelin

In 1999, Scholler and colleagues reported the development of a blood ELISA test that measured "soluble member(s) of the mesothelin/megakaryocyte potentiating factor family" in sera from patients with ovarian carcinoma (16). They named this material SMRP (soluble mesothelin-related peptide) and suggested that it differed from mesothelin. Our group had also been developing a test to measure mesothelin levels in the blood using two new antibodies to mesothelin (MN and MB), and we thought it very likely that SMRP was actually shed mesothelin (17). To demonstrate the nature of the protein shed from cells, we isolated the protein and determined its amino acid sequence. These studies showed that the shed protein is identical to mesothelin and established that SMRP is actually shed mesothelin (18).

Mesothelin levels are elevated in about 50% of patients with mesothelioma and ovarian cancer (17, 19). Unexpectedly, mesothelin levels are not elevated in patients with pancreatic cancer despite the fact that the majority of pancreatic adenocarcinomas highly express mesothelin on the cell surface (20). It is also not clear why mesothelin levels are not elevated in more patients with mesothelioma because the protein is highly expressed in almost all patients with epithelial-type mesothelioma. Possible reasons for the lack of detection of mesothelin in the blood are poor shedding, destruction in the cancer before entry into the blood, barriers to entry into the blood, and small-volume disease. Several ELISAs have also been developed to measure the levels of MPF in human serum (21). MPF levels, like mesothelin levels, are elevated in many patients with mesothelioma and ovarian cancer but are not elevated in patients with pancreatic adenocarcinoma (20).

Although mesothelin is attached to the cell membrane through its glycosylphosphatidylinositol (GPI) anchor and phospholipase C can release the protein from the cell, it seems that phospholipase C does not play a significant role in the physiologic shedding process. Mesothelin shedding is mediated by the sheddase TNF- α converting enzyme, which is a member of the matrix metalloproteinase/a disintegrin and metalloprotease family (22).

Mesothelin Function

Although many studies have been carried out on the possible function of mesothelin, its role in cancer is still unclear and may be cancer type specific. Mice in which the mesothelin gene had been inactivated appeared perfectly normal. They bred normally, had normal blood counts, and grew to normal size (23).

It was shown that cells expressing CA125 bind to cells expressing mesothelin (24). Furthermore the anti-mesothelin antibody, MORAb-009, prevents this binding (25). On the basis of these studies, it was suggested that mesothelin has a role in the spread of ovarian cancer cells in the peritoneal cavity and mesothelioma in the pleural and peritoneal cavity. However, no direct evidence in support of this hypothesis was published, although an interaction of mesothelin and CA125 must occur in patients, because patients treated with the mesothelin-binding antibody MORAb-009 had an elevation in serum levels of CA125 (26).

A variety of studies have been done to evaluate the function of mesothelin. In Eker rats, the development of tuberosus sclerosis-2-induced renal carcinoma was significantly reduced in the absence of a homologue of the *MSLN* gene (27). In pancreatic cancer cells, overexpression of *MLSN* was implicated in significant enhancement of tumor cell growth and migration *in vitro*, but the molecular mechanism that contributes to these phenotypes is not well understood (28). On the other hand, human mesothelioma cells frequently lose mesothelin expression when placed in culture, indicating that mesothelin is not needed for growth of mesothelioma cells, although it may contribute to the aggressive behavior of some types of cancer in animals (29). Recently it was shown that in patients with early-stage lung adenocarcinoma, overexpression of mesothelin is associated with decreased overall survival (30). However, the precise role of mesothelin in carcinogenesis is unclear and is an active area of investigation.

Clinical Trials

Because of the high expression of mesothelin in many malignancies (Fig. 1B), a variety of agents are being developed to target mesothelin. Results of several ongoing clinical trials of immunotherapy agents directed against mesothelin have shown that targeting mesothelin is safe and does not result in toxicity to essential normal tissues. Both antibody-based therapies, as well as mesothelin vaccines, are being investigated.

Immunotoxins

The first mesothelin-directed agent to enter the clinic was the immunotoxin SS1P (Fig. 1C). Because the SS1P contains a bacterial protein, the goals of the phase I trials were to

determine its safety profile, to determine a maximum tolerated dose (MTD), and to assess how many doses could be given before antibodies formed and inactivated the toxin. In one trial, SS1P was given as a bolus infusion over 30 minutes and the dose-limiting toxicity was pleuritis. A second cycle of SS1P could only be given to 10% of patients due to neutralizing antibody formation (31). In another trial, SS1P was given as a continuous infusion for 10 days. Dose-limiting side effects were very similar to those from the bolus infusion trial (32). In neither trial were major responses observed, although there was shrinkage of small-volume disease in some patients.

One factor limiting the activity of large-molecular-weight molecules like immunotoxins and antibody drug conjugates (ADC) is penetration into solid tumors. To overcome this limitation, we performed a clinical trial in which we combined SS1P with chemotherapy. We had shown in mouse studies that chemotherapy, by disrupting tumor cell packing and lowering intratumoral fluid pressure, allowed immunotoxin to reach more cells within the tumor and produced better antitumor responses (33). In the trial, SS1P was combined with pemetrexed and cisplatin for the treatment of chemotherapy-naïve patients with unresectable pleural mesothelioma. Results of this clinical trial showed that SS1P can be safely combined with pemetrexed and cisplatin and both chemotherapy and SS1P could be administered at their MTDs without overlapping toxicity. Out of the 13 patients treated at the MTD, 10 patients had objective partial responses. Although this was a small trial, the objective response rate of 77% was significantly better than the 41% response rate seen in patients treated with chemotherapy alone (34). However, as seen in the phase I clinical trials of SS1P alone, 90% of patients developed neutralizing antibodies to SS1P after only one cycle of SS1P and limited retreatment.

Development of human antibody response to immunotoxins in patients with solid tumors is a significant impediment to their clinical development, and previous efforts to limit their immunogenicity by treatment with single agents such as steroids, cyclophosphamide, cyclosporine, or rituximab have not been successful (35–37). However, we have recently shown that a regimen of two agents, pentostatin and cyclophosphamide, to deplete T and B cells, completely abrogated the development of anti-SS1P antibodies in immune-competent Balb/C mice, which were immunized with SS1P (38).

This strategy of using pentostatin and cyclophosphamide to prevent human antibody response to SS1P was evaluated in a pilot study of heavily pretreated chemotherapy-refractory mesothelioma patients. Eleven patients were enrolled in the study and received pentostatin and cyclophosphamide before SS1P administration. This regimen was well tolerated, and no patient developed opportunistic infections. As predicted, this regimen decreased the development of human antibodies to SS1P; only two of ten patients developed antibodies after one cycle of therapy, which was significantly better than that in prior clinical trials where about 90% of patients developed antibodies after one cycle of therapy (39). Unexpectedly, we observed remarkable antitumor activity in these treatment-refractory patients who had poor prognosis (Fig. 1D). Three of the ten evaluable patients with extensive tumor burden had

durable partial responses, and two of these patients had complete metabolic responses by positron emission tomography scan. All three patients were alive after 27, 25, and 22 months of starting therapy. In addition, two patients who did not initially respond to SS1P had a dramatic antitumor response when treated with chemotherapy to which they had not previously responded. We have recently initiated additional clinical studies in which the pentostatin and cyclophosphamide regimen will be evaluated in more patients with mesothelioma, as well as in patients with pancreatic cancer.

Chimeric anti-mesothelin antibody

MORAb-009 (amatuximab) is a chimeric antibody directed to mesothelin. In preclinical studies, it kills mesothelin-expressing cell lines by antibody-dependent cell-mediated cytotoxicity and also inhibits the interaction between mesothelin and CA-125 (25). In a phase I clinical trial, this antibody was well tolerated and the MTD was established as 200 mg/m² (40). A randomized phase II clinical trial of amatuximab plus gemcitabine versus gemcitabine alone in patients with pancreatic cancer failed to show any advantage to the combination therapy (41). In a nonrandomized clinical trial of patients with advanced unresectable pleural mesothelioma, amatuximab was given in combination with pemetrexed and cisplatin. Although this trial did not achieve its primary objective, improvement in progression-free survival as compared with historical controls, it did show improvement in overall survival with a plateau at the end of the survival curve (42). These data are encouraging for patients with advanced mesothelioma and suggest that this regimen should be evaluated in a randomized clinical trial compared with pemetrexed and cisplatin alone.

Antibody drug conjugates

BAY94-9343 is an ADC in which a humanized immunoglobulin G 1 anti-mesothelin mAb is linked to the maytansine derivative DM4; it is currently undergoing phase I evaluation in patients with advanced solid tumors. BAY94-9343 is given as an intravenous infusion every 3 weeks until disease progression or unacceptable side effects. Preliminary results of this trial show an acceptable toxicity profile, and the MTD has not yet been achieved (43).

Chimeric antigen receptor T-cell therapy

Chimeric antigen receptor (CAR) T cells are engineered to express the Fv portion of a mAb fused to a T-cell receptor. Binding of the CAR to the tumor antigen activates T-cell signaling and results in cell killing. Because early clinical trials of SS1P showed no off target toxicity, there is a growing interest in exploiting mesothelin as a target for CAR T-cell therapy. Currently, two anti-mesothelin CAR T-cell approaches are being pursued (44, 45). Preliminary results of mesothelin-specific CAR mRNA-engineered T cells show that this approach is safe and can induce antitumor responses (46).

Mesothelin tumor vaccines

The only mesothelin tumor vaccine currently in clinical development is CRS-207, which consists of a live-attenuated strain of the bacterium *Listeria monocytogenes* expressing

human mesothelin. The safety of this vaccine was established in a phase I clinical trial of patients with mesothelin-expressing refractory cancers (47). This vaccine has recently been evaluated in a randomized clinical trial of patients with advanced pancreatic cancer. These patients were randomized to six cycles of GVAX (allogeneic pancreatic cancer cell lines secreting granulocyte macrophage colony-stimulating factor) vaccine alone or two cycles of GVAX followed by four cycles of CRS-207 every 3 weeks. A total of 90 patients were enrolled with 61 treated with GVAX/CRS-207 and 29 with GVAX alone. After a median follow-up of 7.8 months, the median overall survival of patients treated with GVAX/CRS-207 was 6.1 months versus 3.9 months for patients treated with GVAX alone ($P = 0.011$; ref. 48). Although a small study, these results are impressive in this study population and need to be validated in a larger phase III setting. CRS-207 is also in early stages of clinical evaluation in patients with mesothelioma and is being tested in a first-line setting in combination with pemetrexed and cisplatin.

Future Directions

Clinical trials of different agents targeting mesothelin have confirmed that it is an excellent target for cancer immunotherapy, and much of the effort is now focused on larger trials to confirm the preliminary hints of antitumor activity seen in patients. Our studies have shown that combining SSIP with

pentostatin and cyclophosphamide can delay antibody formation to SSIP and lead to durable antitumor activity in some patients. We have also used protein engineering to identify and remove B- and T-cell epitopes in SSIP to make a less immunogenic immunotoxin (49, 50). On the basis of these studies, we have recently developed a new immunotoxin in which domain II of PE was deleted and six residues in domain III mutated to alanine. Because the deletion of domain II resulted in a protein that is small and rapidly filtered by the kidney, we collaborated with Roche Pharmaceuticals to replace the Fv with a Fab to make RG7787. Preclinical studies with RG7787 have shown that large doses can be safely given to mice, it has a decreased capacity to cause capillary leak syndrome in rats, and it has significant antitumor activity in mice bearing several types of mesothelin-expressing tumors. RG7787 is expected to undergo phase I clinical evaluation by the end of 2014.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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