Megakaryocyte Potentiation Factor Cleaved from Mesothelin Precursor Is a Useful Tumor Marker in the Serum of Patients with Mesothelioma

Masanori Onda,1 Satoshi Nagata,1 Mitchell Ho,1 Tapan K. Bera,1 Raffit Hassan,1 Richard H. Alexander,2 and Ira Pastan1

Abstract

Purpose: To establish monoclonal antibodies (mAb) against megakaryocyte potentiation factor (MPF) and detect MPF in the blood of patients with mesothelioma.

Experimental Design: Mice were immunized with a purified recombinant human MPF-rabbit-Fc fusion protein and with MPF. Several hybridomas producing mAbs to MPF were established. A double-determinant (sandwich) ELISA was constructed using mAbs to two different epitopes and used to determine if MPF is present in the serum of patients with mesothelioma.

Results: We established seven anti-MPF mAbs whose topographical epitopes were classified into three nonoverlapping groups. All the mAbs reacted with recombinant MPF protein by ELISA. One of the mAbs detected MPF and the mesothelin precursor protein containing MPF in cell lysates on Western blotting. A sandwich ELISA using mAbs to two different epitopes was constructed and used to measure the presence of MPF in the media of various mesothelin-expressing cancer cell lines and in human serum. The ELISA showed that MPF levels were elevated in 91% (51 of 56) of patients with mesothelioma compared with healthy controls. Furthermore, serum MPF fell to normal levels in two patients after surgery for their peritoneal mesothelioma.

Conclusions: Using new mAbs to MPF, we showed that MPF is secreted by several mesothelioma cell lines and is frequently elevated in the blood of patients with mesothelioma. Measurement of MPF may be useful in following the response of mesothelioma to treatment.

Megakaryocyte potentiation factor (MPF) is a 33-kDa soluble protein that is produced by proteolytic cleavage of the 69-kDa mesothelin precursor protein (1, 2). The other cleavage product is mesothelin, a cell membrane–associated 40-kDa glycosyl-phosphatidylinositol-linked glycoprotein (1). Mesothelin is present on normal mesothelium but is highly expressed on mesotheliomas and other tumors, including ovarian and pancreatic cancers (3–8). The biological function of mesothelin and MPF is not clear. MPF was originally identified as a polypeptide from the culture supernatant of a pancreatic cancer cell line and was shown to stimulate the megakaryocyte colony-forming activity of murine interleukin-3 in mouse bone marrow cell cultures (9). However, mesothelin knockout mice, which do not produce the mesothelin precursor protein encoding both MPF and membrane-bound mesothelin, showed no discernible phenotype and had normal platelet counts (10). Recently, mesothelin has been shown to bind to CA125/ MUC16 that is present on many ovarian cancer cells, and it was suggested that mesothelin might have a role in the metastatic spread of ovarian cancer in the peritoneal cavity (11).

Malignant mesothelioma is a highly aggressive tumor of serosal cavities that arises from the mesothelial cells of the pleura, peritoneum, or pericardium (12). Although the incidence of mesothelioma in the United States seems to be leveling off, its incidence is still increasing in other countries (13–15). Although some advances have been made in the treatment of this disease, the prognosis of patients is still poor with a median overall survival of about 9 to 12 months (12). However, surgery can lead to improved survival in the small percentage of patients who are diagnosed at an earlier stage (16). A sensitive tumor marker that allows early diagnosis of mesothelioma in people at risk for this disease would, therefore, be clinically useful.

Because virtually all epithelial mesotheliomas overexpress mesothelin, we wanted to determine if MPF, a proteolytic fragment of the mesothelin precursor protein, is shed into the blood stream of patients with mesothelioma. To measure MPF that may be released into the circulation, we generated monoclonal antibodies (mAb) specifically directed to MPF. Using anti-MPF mAbs that react with different epitopes on the MPF protein, we developed a sandwich ELISA to measure the MPF level in human serum. Our results show elevated MPF...
levels in the sera of patients with mesothelioma compared with healthy donors. Our findings also suggest that measurements of MPF in the blood could be potentially useful to monitor treatment response.

Materials and Methods

**Human serum samples.** Seventy serum samples from healthy blood donors obtained from Bioreclamation, Inc. (Hicksville, NY) were used as controls. The median age of healthy volunteers was 39 years (range, 18-65 years; 36 males and 34 females). Sera from 56 patients with mesothelioma who were treated on Surgery Branch protocols at the National Cancer Institute. These patients underwent tumor debulking with intraoperative continuous hyperthermic peritoneal perfusion with cisplatin (18). The patients were treated on an Institutional Review Board–approved protocol.

**Generation of MPF-rFc and MPF proteins.** MPF was expressed as a fusion protein with rabbit IgG Fc (rFc) in HEK293T cells as previously described (19). The DNA fragment encoding MPF protein (amino acids 1-291) was PCR amplified from the plasmid pMH107 (Genbank accession no. AY744392; ref. 20) and was inserted into the rFc expression plasmid based on pDNA3 (Invitrogen, Carlsbad, CA). Mesothelin (amino acids 296-599) was also expressed as a fusion protein with rFc. MPF protein was released from the MPF-rFc by Prescision protease treatment (Amersham Biosciences, Piscataway, NJ) that cut the recognition site between MPF and rFc. After cleavage, the Precision protease that is a fusion protein with glutathione S-transferase was removed by glutathione-Sepharose (Amersham Biosciences) column. The purity of the prepared MPF protein was shown by SDS-PAGE analysis (Fig. 1).

**Generation of mAbs against MPF protein.** Ten female mesothelin-deficient mice (6-8 weeks old) were immunized thrice with the MPF-rFc fusion protein (50 μg in 100 μl PBS) i.p. at intervals of 2 weeks. All animals were maintained in accordance with institutional guidelines. Three days before fusion, MPF protein (20 μg) was injected i.p. Cell fusions of the splenocytes and the SP2/0 cells, selection of the hybridomas, and screening for mAb production by an indirect ELISA source, Camarillo CA) at 1:20,000 for 1 hour. MPF and mesothelin antibodies for 2 hours at the following concentrations: 2 μg/ml anti-MPF mAb (MPF44). 1 μg/ml anti-mesothelin mAb (MN), and 1 μg/ml anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected by a horseradish peroxidase–conjugated secondary antibody and goat anti-mouse immunoglobulin G (Bio-source, Camarillo CA) at 1:20,000 for 1 hour. MPF and mesothelin proteins were visualized by the enhanced chemiluminescence kit (Amersham Biosciences).

**PNGase F digestion.** Whole-cell lysates (40 μg) or purified recombinant proteins (10 μg) were digested with PNGase F (New England Biolabs, Ipswich, MA) for 2 hours at 37°C as instructed by the manufacturer. N-linked deglycosylation of cell lysates was then examined by Western blot analysis.

**Western blot using recombinant MPF protein.** The reactivity of the anti-MPF mAbs to SDS-denatured antigen was tested in a Western blot analysis. MPF-rFc, bovine serum albumin, and MPF (50 ng of each) were separated by 4% to 20% Tris-glycine SDS-PAGE and transferred onto nitrocellulose filters. The filters were incubated with primary antibodies for 2 hours at the following concentrations: 2 μg/ml anti-MPF mAb (MPF44). 1 μg/ml anti-mesothelin mAb (MN), and 1 μg/ml anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected by a horseradish peroxidase–conjugated secondary antibody and goat anti-mouse immunoglobulin G (Bio-source, Camarillo CA) at 1:20,000 for 1 hour. MPF and mesothelin proteins were visualized by the enhanced chemiluminescence kit (Amersham Biosciences).

**Topographical epitope mapping.** Topographical epitopes of anti-MPF mAbs were determined by the assessment of the mutual competition of all possible pairs of mAbs in label-free competitive ELISA (22). Briefly, an immune complex formed by incubation of MPF-rFc and a mAb (competitor) is tested for binding to another mAb (indicator) that had been captured by goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) on the plate. If the topographical epitopes of these two mAbs are the same, MPF-rFc will not be able to bind to the indicator mAb because the competitor mAb has already occupied the
The expression of megakaryocyte potentiation factor (MPF) was examined in various cell lines. Cells were cultured in medium supplemented with fetal bovine serum and sodium azide, and the supernatants were collected for further analysis.

### Sandwich ELISA

Purified MPF (4 μg/mL) was absorbed onto ELISA plates (50 μL/well) at 4°C overnight. The plates were then blocked with blocking buffer (25% DMEM, 5% FCS, 25 mM HEPES, 0.5% bovine serum albumin, 0.1% azide = 100 μL/well) for 30 minutes at room temperature. After washing, diluted samples or standards were added to the plates (50 μL/well). After overnight incubation at 4°C, the plates were washed twice and incubated with biotinylated mAb MPF25 (0.5 μg/mL in blocking buffer). Plates were then washed twice and incubated with 1:2,000 dilution of streptavidin-horseradish peroxidase. The enzyme reaction was allowed to proceed at room temperature for 20 minutes. The absorbance at 450 nm was measured with the reference at 600 nm.

### Results

#### Generation of a recombinant MPF-rFc fusion protein and generation of anti-MPF mAbs

The mesothelin precursor protein contains 622 amino acids and is composed of MPF (amino acids 1-295) and mesothelin (amino acids 296-622). To obtain mAbs that recognize MPF, we prepared a MPF-rFc fusion protein composed of the full-length MPF (amino acids 1-291) connected by a peptide linker containing a Prescision protease site to an Fc fragment of rabbit IgG1. Purified MPF was prepared by treating the fusion protein with Prescision protease (Fig. 1).

Mesothelin-deficient mice were used for the immunization because it seemed possible they would respond to MPF immunization better than the wild-type mice because their immune system had not been educated to the mouse homologue of human mesothelin. To try and obtain as many different mAbs as possible, 10 mesothelin-deficient mice were immunized with MPF-rFc. All mice showed a high titer against MPF (>1:10,000). From four mice, 56 hybridomas secreting mAbs specifically reacting with MPF were established (32 IgG1, 10 IgG2a, 10 IgG2b, 2 IgG3, and 2 IgM). Seven mAbs showing the highest ELISA signal were further characterized (Table 1).

### Table 1. Characteristics of anti-MPF mAbs

<table>
<thead>
<tr>
<th>Name</th>
<th>Isotype</th>
<th>Titer (mL/μg)</th>
<th>Topographic epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF25</td>
<td>γ1,κ</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td>MF44</td>
<td>γ1,κ</td>
<td>2,732</td>
<td>2</td>
</tr>
<tr>
<td>MF45</td>
<td>γ1,κ</td>
<td>1,368</td>
<td>2</td>
</tr>
<tr>
<td>MF46</td>
<td>γ2a,κ</td>
<td>1,024</td>
<td>2</td>
</tr>
<tr>
<td>MF48</td>
<td>γ1,κ</td>
<td>1,332</td>
<td>2</td>
</tr>
<tr>
<td>MF49</td>
<td>γ2a,κ</td>
<td>129</td>
<td>3</td>
</tr>
</tbody>
</table>

*The reciprocal of the mAb concentration needed to attain an absorbance of 0.2 in indirect ELISA in the same plate. Values are equal to the titers of 1 μg/mL of mAb solution. In the ELISA, MPF protein was coated on the plate, and various concentrations of mAbs were added. The titer was calculated from the titration curves by a four-variable logistic curve fitting of the data. All mAbs did not react to a negative control (recombinant mesothelin-Fc) in a similar ELISA.

Topographic epitopes determined in a competitive ELISA shown in Table 2. Three primary groups of epitopes (1, 2, and 3) were identified.
mesothelin precursor protein (72 kDa). In samples not treated with PNGase F, the anti-MPF mAb detects a 72-kDa protein that also reacts with an antibody to mesothelin, indicating this is the mesothelin precursor protein (Fig. 2B). There is also a smaller band (40 kDa) that reacts with only the antibody to mesothelin. A protein the size of MPF was not detected within the cell, indicating that MPF was released after its precursor reached the cell surface. In cell extracts treated with PNGase F, the size of the band reacting with both antibodies was reduced to 50 kDa, consistent with deglycosylation. The size of the band reacting only with the antibody to mesothelin was also reduced. Mesothelin contains three potential N-linked glycosylation sites, whereas MPF has only one site (1). This experiment shows that the anti-MPF antibody is useful for detecting the full-length mesothelin precursor protein in cells. There is no detectable free MPF in cells, indicating it is released after the precursor reaches the cell surface.

**Topographical epitope mapping of anti-MPF mAbs.** To construct a sandwich ELISA that can measure MPF in tissue culture supernatants and in the blood of patients, we need to use mAbs that react with different epitopes on MPF. To clarify the topographical epitopes of the mAbs, a competitive ELISA was carried out as described in Materials and Methods (label-free competitive ELISA; ref. 22). As shown in Table 2, the competition percentages of competitors varied from 4 (no competition) to 119 (complete competition), depending on the contribution of indicator and competition of the mAbs. When antibody binding is reduced by >40% for one pair of mAbs in a mutual manner, the two mAbs are judged to be located in the same topographical epitope group. This method revealed that the mAbs can be divided into three epitope groups (epitopes 1-3; Table 2). This result indicates that mAbs from different groups can bind to MPF at the same time. To develop a sandwich ELISA, we chose three mAbs (MPF25, MPF44, and MPF49) with the highest titers in each epitope group (Table 1). Six different ELISAs were constructed using different combination of mAbs, and we evaluated the linearity and sensitivity of each of them. We chose the pair of mAbs MPF25 and MPF49 to construct a sandwich ELISA because this combination showed the best performance (data not shown).

**Measurement of MPF by ELISA.** A sandwich ELISA was developed using the anti-MPF mAbs MPF25 and MPF49. The standard curve using purified recombinant MPF is shown in Fig. 3.
Fig. 3. Reproducibility of this sandwich ELISA is in an acceptable range over the range of 1 to 8 ng/mL of MPF (±10% of CV, n = 9). The assay was then used to determine if stably expressing mesothelin-transfected A431/K5 cells that express the full-length mesothelin gene and are known to contain mesothelin protein on their surface release MPF into the medium. The data in Fig. 4 show that A431/K5 cells secrete MPF in a time-dependent manner over 6 days. As expected, no MPF was detected in the supernatants of the epidermoid carcinoma cell line A431 that does not express the mesothelin gene (Fig. 4). Finally, the assay was used for the detection of the MPF from the native cancer cells. The production of MPF by various cancer cell lines derived from different cancer types was measured using cells growing in 96-well culture plates. As shown in Table 3, three mesothelin-positive cell lines (HeLa, A1847, and IGROV1) secrete MPF. No MPF was detected in the media from mesothelin-negative OHS-M1 osteosarcoma and A431 epidermoid carcinoma cells.

MPF in serum of patients with mesothelioma. A total of 56 sera were obtained from patients with a histopathologic confirmation of mesothelioma. Sera from healthy volunteers (n = 70) with a median age 39 years were used to determine the cutoff for positivity for this sandwich ELISA. The average absorbance ±SD from healthy volunteers using a sandwich ELISA was 0.019 ± 0.005. Absorbance values of a 1:10 diluted serum that exceeded the mean absorbance value of sera from healthy donors by 3 SDs were considered positive. This cutoff based on absorbance values is similar to what has been described for other ELISA systems (19, 25). Thus, serum samples with absorbance values of ≥0.034 were considered to have elevated MPF levels. As shown in Fig. 5, MPF was not detected in sera from healthy donors (n = 70). However, MPF was detected in the sera from 91% of patients with mesothelioma (51 of 56 patients). The average absorbance ±SD of the mesothelioma sera was 0.76 ± 0.63. These two groups were compared by nonparametric Mann-Whitney test. The P revealed that the difference between these groups was statistically very significant (P < 0.0001).

Effect of surgical treatment on serum MPF levels in patients with peritoneal mesotheliomas. To determine if serum MPF levels can be used to measure treatment response, we measured MPF levels in four patients with peritoneal mesothelioma before and after surgical tumor debulking. The clinical characteristics of the patients are summarized in Table 4. Three patients (A, B, and C) with mesothelin-expressing mesotheliomas had elevated serum MPF levels before therapy (Fig. 6). In patients A and B, the serum MPF levels decreased immediately after surgery. Patient C was found to have extensive tumor spread, and only limited tumor removal (~10%) could be

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**Table 3. Mesothelin expression and MPF protein in the supernatant of cancer cell lines**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cells</th>
<th>Anti-mesothelin reactivity by FACS*</th>
<th>MPF in supernatant (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian cancer</td>
<td>A1847</td>
<td>++</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>IGROV1</td>
<td>+</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>HeLa</td>
<td>++</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Epidermoid cancer</td>
<td>A431</td>
<td>–</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>OHS-M1</td>
<td>–</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Log geometric mean of fluorescence intensity: the difference between mesothelin and background (2.0) ++ ≥ 1.0; 1.0) + > 0; – = 0).  
1 Culture supernatants on day 6. On day 0, 6 × 10^5 cells were seeded on 96-well culture plates.

---

**Table 4. Clinical characteristics of the patients with mesothelioma**

<table>
<thead>
<tr>
<th>Patient* Gender/ Age</th>
<th>Pathology</th>
<th>Mesothelin expression</th>
<th>Ascites</th>
<th>Debulked (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M/68</td>
<td>Epithelial</td>
<td>Yes</td>
<td>Yes 95</td>
</tr>
<tr>
<td>B</td>
<td>M/63</td>
<td>Epithelial</td>
<td>Yes</td>
<td>Yes 70</td>
</tr>
<tr>
<td>C</td>
<td>F/31</td>
<td>Epithelial</td>
<td>Yes</td>
<td>Yes 10</td>
</tr>
<tr>
<td>D</td>
<td>M/46</td>
<td>Biphasic</td>
<td>No</td>
<td>No 90</td>
</tr>
</tbody>
</table>

* These samples were used for MPF assay (Fig. 6).  
1 Estimated by immunohistochemistry. Tumors were considered mesothelin expression if >90% of tumor cells had cell surface expression of mesothelin.
done. In this patient, post-surgical serum MPF values did not change significantly from the baseline. Patient D had a mesothelin-negative tumor, and no MPF was detected before or after surgery. These results indicate that MPF may be useful to monitor response to therapy.

Discussion

Using mesothelin-deficient mice and immunization with recombinant MPF-Fc fusion protein, we generated several mAbs that specifically recognize MPF. Next, we did topographical epitope mapping of these anti-MPF antibodies and determined that they recognize three different epitope groups of the MPF protein. Using mAbs MPF25 and MPF49 that bind to two different epitopes on MPF, we developed an ELISA to measure MPF in cell culture supernatants and human serum.

Using our sandwich ELISA, we evaluated MPF levels in culture supernatant of several cell lines. Elevated MPF levels were noted in ovarian and cervical cancer cell lines that were mesothelin positive by FACS analysis. In contrast MPF levels in the culture supernatant were absent in mesothelin-negative epidermoid carcinoma and osteosarcoma cell lines. These results show that MPF is indeed shed into the media of cell lines that express mesothelin (Table 3).

To determine whether MPF is shed into blood, we analyzed serum samples from 70 normal healthy donors and 56 patients with advanced malignant mesothelioma. We choose to use serum samples from patients with mesothelioma because virtually all epithelial mesotheliomas overexpress mesothelin (26). Our results showed that MPF levels were elevated in 91% of these patients. In addition, using serum samples from patients with peritoneal mesothelioma who were undergoing debulking surgery, MPF levels decreased in patients who had optimal tumor debulking. In the current study, we used samples from patients with late-stage disease who came to the National Cancer Institute to be evaluated for a phase I clinical trial. In the future, we plan to test serum from patients with early-stage mesothelioma and other mesothelin-expressing cancers. We also plan to measure MPF levels in other groups of individuals, including humans exposed to asbestos and individuals of different ages.

The cDNA encoding mesothelin and MPF was identified by two different groups using different approaches. Our group identified mesothelin as the antigen recognized by mAbK1, which we obtained by immunizing mice with the ovarian cancer cell line OVCAR-3 (1, 3). On the other hand, MPF was identified by Yamaguchi et al. from the culture supernatant of a pancreatic cancer cell line and had some megakaryocyte potentiating activity in mouse bone marrow cultures (2, 9).

Both the MPF and mesothelin are encoded by the same gene that produces a precursor protein of 69 kDa. This precursor protein has a furin cleavage site (RPRFRR) between amino acids 288 and 293. Cleavage at this furin site results in the release of the MPF from the cell membrane by a glycosylphosphatidylinositol anchor (17). We have recently shown that cell-bound mesothelin can also be released into the blood of patients with mesothelioma and ovarian cancer and may be a useful marker for mesothelin-expressing cancers (27). The release of mesothelin from the cell membrane is most likely mediated by phospholipase C or D or other proteases because glycosylphosphatidylinositol-linked proteins are frequently shed from the cell surface by such proteases (28, 29). In addition, soluble mesothelin-related proteins have been described in serum of patients with ovarian cancer and mesothelioma (25, 30).

Although MPF is presumed to be released by proteolytic cleavage of the 69-kDa precursor protein by furin, its detection in human blood has been hampered by the lack of antibodies that specifically recognize it. Using anti-MPF mAbs that we generated, we developed an ELISA to measure it in human serum. Elevated MPF levels were noted in 91% of serum samples obtained from patients with advanced mesothelioma. Because mesothelin is expressed by several other cancers, especially ovarian and pancreatic cancer, it will be important to study whether elevated MPF levels are also present in serum from patients with these cancers. MPF may also be a useful marker for these cancers. Additional studies are also needed to determine if serum MPF levels can be used as a surrogate marker for response to therapy. Furthermore, it will be important to correlate serum MPF levels with serum mesothelin levels in patients with mesothelin-expressing cancers.

Acknowledgments

We thank Dr. Mark Willingham (Department of Pathology, Wake Forest University, NC) for pathologic diagnoses and members of the Laboratory of Molecular Biology for useful discussions.

Fig. 6. Serum MPF levels of patients with peritoneal mesothelioma before and after surgical resection of the tumors. Using sandwich ELISA, MPF level was indicated as absorbance. Four patient samples were used in this assay with 1:10 dilution. Patient A (●), patient B (▲), and patient C (■) had epithelial mesothelioma. Patient D (○) had biphasic type. Patients A, B, and C had mesothelin expression in immunohistochemistry, but patient D did not have mesothelin expression. All periods of patient D sera were negative (A < 0.034).

References


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