# Mesothelin Variant 1 Is Released from Tumor Cells as a Diagnostic Marker

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## Abstract

**The mesothelin family comprises (at least) three variants and includes the precursor for megakaryocyte potentiating factor (MPF). Assaying soluble mesothelin-related protein (SMRP) molecules in serum and other body fluids from patients with certain cancers can provide diagnostically useful information. We have constructed fusion proteins of mesothelin variants 1, 2, and 3, made monoclonal antibodies, and investigated the binding specificity of these and three previously generated monoclonal antibodies to each of the three mesothelin variants. According to flow cytometry, the molecule that is most frequently expressed at the surface of cells from ovarian carcinomas and certain other tumors is mesothelin variant 1. Similarly, SMRP released into ascites from a patient with ovarian carcinoma was shown to have a molecular weight of ~40 kDa and, according to sequencing, to be variant 1. A published sandwich ELISA was shown to detect variants 1 and 3 and to be much more sensitive than a newly constructed ELISA, which detects only variant 3, the former being positive in 28 of 41 (68%) sera from patients with ovarian cancer as compared with 6 of 41 sera (15%). A standard curve was constructed to measure SMRP with a limit of detection of 200 pg/mL to facilitate future quantitative studies.** (Cancer Epidemiol Biomarkers Prev 2006;15(5):1014–20)

## Introduction

Some tumors (e.g., most ovarian cancers) are curable when discovered early whereas relapses are common when they are detected late even when temporary remissions are achieved. Serum markers are frequently used to aid the diagnosis of several types of cancer and to monitor responses to treatment (1-3). To be detectable in assays of sera or other body fluids, a marker needs to be soluble.

Pastan's group showed that most ovarian cancers express mesothelin, a 40-kDa glycoprotein, and reported that it was stably expressed at the cell surface and not released into serum like CA125 and, therefore, was a suitable therapeutic target (4-7). Later studies indicated that mesothelin may have a role in cell adhesion and possibly in cell-cell recognition and signaling by interacting with CA125 (8). A precursor 69-kDa protein (6) is synthesized and forms two proteins, the membrane-bound mesothelin and a soluble protein, megakaryocyte potentiating factor (MPF), which was first reported by Kojima et al. (9) and has a molecular weight of ~30 kDa.

In 1999, Scholler et al. (10) described a monoclonal antibody (mAb), 569, which binds to an ~40-kDa molecule with an $NH_2$-terminal sequence similar to that reported by Pastan's and Kojima's groups, and is overexpressed in ovarian cancers and certain other tumors. They showed that this molecule was released into culture supernatants, sera, and malignant effusions from patients with certain tumors. A member of the mesothelin family that had an 82-bp insert was identified and indirect evidence suggested that this molecule was soluble. In view of published evidence that mesothelin is not soluble, Scholler et al. (10) speculated that this is the molecule that is released from certain tumor cells (e.g., into serum). A double determinant (''sandwich'') ELISA was constructed by using two mAbs, 569 and 4H3, which recognize two different epitopes on the same antigen. Applying this assay, soluble mesothelin-related molecules were detected in sera and malignant effusions from patients with ovarian carcinoma (10). Evidence has been published indicating that an ELISA using mAbs 569 and 4H3 to measure soluble mesothelin-related protein (SMRP) holds promise for detection of mesothelioma (11), for which there was no previous good biomarker, and that it favorably complements CA125 for detection of ovarian carcinoma (12).

Subsequent studies have shown that there are at least three mesothelin variants (13, 14): variant 1, variant 2, which has a 24-bp insert, and variant 3, which has an 82-bp insert. We have made fusion proteins of each of these three variants, analyzed the mAbs used for the published sandwich ELISA (10), obtained and analyzed additional mAbs, as well as constructed and evaluated an ELISA specific for variant 3. Mesothelin variant 1 was found to be the one primarily expressed at the surface of cells from certain tumors and to be released into body fluids with a molecular weight of ~40 kDa whereas variants 2 and 3 are expressed and released much less frequently.

## Materials and Methods

**Construction and Expression of Fusion Proteins.** A technique introduced by Capon et al. (15) was applied to construct fusion proteins with an immunoglobulin G (IgG) tail, as previously described for another ovarian cancer marker, HE4 (16). Fusion proteins were designed that include the leader peptide of human HE4 protein (GenBank accession no. AY212888) and the Fc domain of mouse or human IgG. The MR3'300 fragment was amplified from a

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**Note:** N.Y. Sardesai and T. Verch are employed by Fujirebio Diagnostics, Inc., which makes diagnostic tests for cancer. The other authors have no commercial associations that might cause a conflict of interest. Serum samples were provided by Fujirebio Diagnostics, Inc. and had been obtained according to approved procedures. All procedures involving animals were approved by the animal committee at the Pacific Northwest Research Institute, where the animal work was carried out. Determination of antibody avidity was done at Protein Interaction Facility, University of Utah under the Directorship of Dr. D. Myszka.

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plasmid containing mesothelin sequence (GenBank accession no. AF180951) with forward primer (5′-ACCGGTGCAGGTGGGCGGGGCGGCCAGGCC-3′) and reverse primer (5′-GTTGTTAGATCTCGTGTCTGCAGGGGGCATGTT-3′) using ExTaq DNA polymerase (TaKaRa Shuzo Biomedical, Otsu, Shiga, Japan). PCR products were cloned into the pCR2.1-TOPO vector and verified by DNA sequencing. The MR3′300 insert was restriction digested with *Age*I and *Bgl*II and cloned into *Age*I and *Bam*HI sites of the mammalian expression vector pD18-HE4-LP, a derivative of pD18-HE4 (16) with an *Age*I restriction enzyme site. Constructs with an immunoglobulin tail of either mouse origin (MR3′300-mIgG) for immunization of mice or of human origin (MR3′300-hIgG) for screening of hybridomas were initially transferred into COS cells by DEAE-dextran transient transfections as described (17). Culture supernatants were harvested after 72 hours, immunoprecipitated with protein A-agarose, and then screened by SDS-PAGE and Western blot analysis. CHO-DG44 cells (18) were used to construct stable lines expressing high levels of the fusion proteins of interest, as previously described for HE4 (16).

**Generation of mAbs.** BALB/c mice were immunized six times with MR3′300-mIgG fusion protein, their spleen cells were fused with mouse myeloma cells, and hybridomas were screened for production of mAbs binding to MR3′300-hIgG, as previously described (16). mAbs 1A610, 2B10, and 8C8 were generated and screened against the fusion proteins constructed from mesothelin variant 1, 2, or 3, respectively, as summarized in Table 1.

**Cell Lines.** Human ovarian carcinoma lines that were used in flow cytometry have been previously described (19) and lines were similarly established from mesothelioma and pancreatic carcinoma. The T51 lymphoid cell line was used as a control.

**Biosensor Studies of ''569 Reactive Antigen'' Binding to mAbs 4H3, 569, 1A6-10, and 2B10.** Using standard amine coupling chemistry, surfaces of rabbit anti-mouse Fc (Biacore, Uppsala, Sweden, code BR-1005-14, lot no. 1140659) were constructed on all four surfaces of a standard CM5 chip. Levels ranging between 2,500 and 3,000 relative units were obtained. mAbs were captured separately on different flow cells having anti-mouse Fc surfaces. Analyte was then injected over these flow cells. Finally, acid was used to regenerate these surfaces so that the binding cycle could be repeated. All experiments were done at 25°C in HBS buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl] supplemented with 005% P20 (Biacore, code BR-1000-54) and 200 μg/mL bovine serum albumin (fraction V, Sigma, St. Louis, MO). 569 reactive antigen (lot 55IV) was tested using 1/50th dilution of the stock provided (10.1 μmol/L) and four 3-fold serial dilutions thereof. For all experiments, analyte association and dissociation times were 420 and 600 seconds, respectively, using flow rates of 20 μL/min. Data were processed and fitted to simple 1:1 binding models using the software program Scrubber.

**Purification and Analysis of SMRP from Human Ascites.** mAb 569 was immobilized on activated sepharose (prepared by Rockland Immunochemicals, Gilbertsville, PA) and soluble 569-reactive proteins were purified from ascites of an stage IV ovarian cancer patient by immunoaffinity chromatography. Bound antigen was eluted using Gentle Elution Buffer (Pierce, Rockford, IL). Peak fractions were tested by ELISA for the presence of SMRP, pooled, dialyzed (3.5 kD Slide-A-Lyzer cassettes, Pierce) against PBS, and concentrated using Amicon Ultra 4 centrifugational filters with a 10-kDa molecular weight cutoff (Millipore, Billerica, MA).

Purified protein was analyzed by SDS-PAGE. Parallel gels were run for (*a*) staining by Coomassie (GelCode Blue, Pierce) or (*b*) transfer onto nitrocellulose membrane and Western blot. The membrane was blocked in PBS + 4% Carnation milk + 0.05% Tween 20 and subsequently exposed to mAb 569-horseradish peroxidase conjugate in the same buffer. Bound antibody was visualized by development with TMB membrane substrate.

Bands that were reactive with mAb 569-horseradish peroxidase conjugate on Western blot were cut out from a parallel Coomassie stained gel. Proteins were analyzed by microcapillary reverse-phase high-performance liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer at the Harvard Microchemistry Facility. Tandem mass spectra were correlated with known sequences using the algorithm Sequest developed at the University of Washington (20) and programs were developed at the Harvard Microchemistry Facility (21), and then reviewed and confirmed manually for consensus with known proteins.

**Flow Cytometry.** Cultured cells were suspended and studied for cell-surface expression of SMRP using a described technique (19) and testing different anti-SMRP mAbs in parallel with a negative control mAb. They were analyzed on a Beckman Coulter XL flow cytometer using System II software.

**ELISA Assays.** Double determinant (sandwich) ELISAs were applied to measure soluble SMRP, detected by measuring absorbance in an ELISA reader (Spectracount microplate photometer; Packard, Palo Alto, CA). One assay, using mAbs 569 and 4H3 that bind to two spatially distinct epitopes of SMRP, was previously described (10) and applied to study sera from patients with mesothelioma (11) or ovarian cancer (10, 12). As described under Results, it detects mesothelin variants 1 and 3. Another ELISA was constructed as part of this study (see Results) to detect mesothelin variant 3. All assays were done on coded samples. A positive value was taken to be >0.200, which is 3 SD above the mean absorbance measurement at 450 nm for healthy controls. All assays included a negative and a positive control on the same ELISA plate. The negative control serum sample (no signal at a dilution of 1:40) was obtained from a healthy volunteer whereas the positive control sample (absorbance >0.2 at a dilution of 1:1,280) was obtained from a patient with ovarian carcinoma.



**Table 1. Summary of mAbs to mesothelin variants**

| Name | Mice immunized against | Binding specificity |
|---|---|---|
| 569 | Human ovarian cancer cells (10) | Mesothelin variants 1 and 3 |
| 4H3 | Antigen purified from ascites via immunoadsorption* (10) | Mesothelin variants 1, 2, and 3 |
| 1A6-10 | Antigen purified from ascites via immunoadsorption* (10) | Mesothelin variant 1 |
| 2B10 | Mesothelin variant 2 fusion protein | Mesothelin variant 2 |
| 8C8 | Mesothelin variant 3 fusion protein | Mesothelin variant 3 |
| 4A10 | Mesothelin variant 3 fusion protein | Mesothelin variant 3 |
| 13H5 | Mesothelin variant 3 fusion protein | Mesothelin variant 3 |

NOTE: All the mAbs are mouse IgG1.
*mAbs 4H3 and 1A6-10 were generated by immunizing mice with antigen purified from malignant cells in the ascites of a patient with ovarian carcinoma by immunoadsorption on a column made with mAb 569.

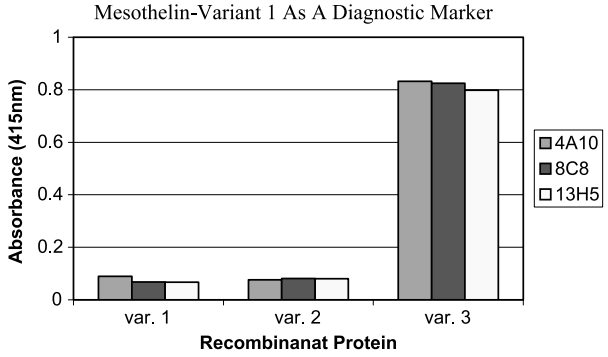


**Figure 1.** Binding of three different mAbs raised against variant 3 to each of the three mesothelin variants as assayed by an ELISA. The mAbs were added to wells, onto the bottom of which fusion proteins of the three mesothelin variants had been attached. There was no binding to the unrelated, similarly constructed fusion protein HE4 (16).

## Results

**Generation of mAbs to Mesothelin Variants and Analysis of Specificity for Mesothelin Variants.** When fusion proteins of mesothelin variants 1, 2, and 3 became available for testing, our first step was to do binding assays with three already existing anti-mesothelin mAbs: 569, 4H3, and 1A6-10 (10). We found that mAb 569 binds to mesothelin variants 1 and 3, mAb 4H3 binds to all three variants, and mAb 1A6-10 exclusively recognizes variant 1. Based on these findings, we attempted to generate mAbs that would exclusively recognize variant 2 or 3 with emphasis on mAbs to variant 3.

Figure 1 presents the results from binding assays with three mAbs (4A10, 8C8, and 13H5) that were generated against variant 3 and the results of all binding assays are summarized in Table 1. The published ELISA (10), which uses mAbs 569 and 4H3, detects variants 1 and 3, but not variant 2.

**Measurement of Antibody Avidity.** Figure 2 shows processed and fit data of analyte 569 reactive antigen binding to mAbs 1A6-10, 569, and 4H3.

The kinetic parameters derived from these fits with 569 reactive antigen as analyte are as follows with respect to $k_a$ ($M^{-1}$ $s^{-1}$), $k_d$ ($s^{-1}$), and $K_d$ (nmol/L), respectively: 1.44(3)e5, 2.45(3)e−4, and 1.70(2) for 1A6-10; 3.02(6)e5, 3.4(4)e−4, and 1.1(9) for 569; and 2.837(3)e5, 1.5(3)e−5, and 0.052(9) for 4H3, where the numbers in brackets denote SD in the last significant digit.

The fits shown in Fig. 2 are high, affirming the validity of the experimental strategy employed to analyze the binding interaction of these mAbs with antigen. For some of the fittings, sensorgrams from the highest or two highest concentrations of analyte injected had to be omitted as these sensorgrams were difficult to fit a simple 1:1 model. This may have to do with possible aggregation of analyte at higher concentrations or, conversely, an increased frequency at these concentrations of bivalent analyte cross-linking captured mAbs. In either case, the effect would be a deviation from simple 1:1 model. The analyte:mAb interaction for mAb 2B10 to 569 reactive antigen could not be assessed due to insufficient captures of this mAb.

**Sequencing of SMRP Isolated from Ascites.** Western blot analysis of mAb 569 affinity-purified protein from ascites revealed a diffuse band at 40 to 45 kDa. This band was excised from a parallel SDS-PAGE gel, digested with trypsin, subjected to high-performance liquid chromatography-mass spectrometry, and resulting peptides were aligned to the reported sequence (Fig. 3A). The peptides mapped to mesothelin variants 1 and 2 (Fig. 3B). Although variant 3 may be present as well, no peptides mapped to the COOH terminus of the proteins beyond amino acid 297 thereby not allowing further conclusions. It seems likely, however, that this part of the sequence is missing from the isolated proteins because no peptides were recovered from this part of the protein whereas most of the remaining sequence could be determined. Both variant 1 and variant 2 were isolated as soluble proteins from ascites whereas the full-length precursor at 69 kDa was not detected. The $NH_2$-terminal sequence of both isoforms started with ''AREIDESL...,'' which is different from the predicted $NH_2$ terminus. Again, the lack of peptides recovered from this region of the protein does not allow conclusions on the precise start of the protein. A previously reported change from aspartic acid to asparagine at position 110 was confirmed by mass spectrometry.

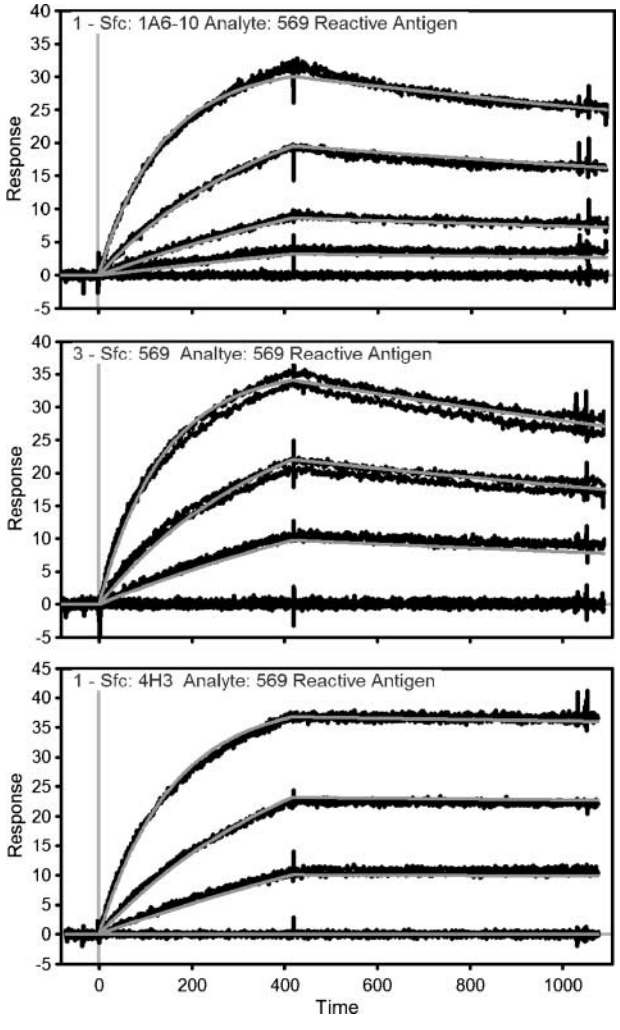


**Figure 2.** 569 reactive antigen binding to captured 1A6-10, 569, and 4H3. Processed data (*black*) of 569 reactive antigen binding to captured 1A6-10, 569, and 4H3 and the fits (*orange*) of a simple 1:1 binding model to this data. Sensorgrams of injected analyte at only four of the five concentrations tested were used to fit 569 reactive antigen binding to 1A6-10 (highest concentration omitted). Sensorgrams of injected analyte at three of the five concentrations tested were used to fit 569 reactive antigen binding to 569 and 4H3 (two highest concentrations omitted).

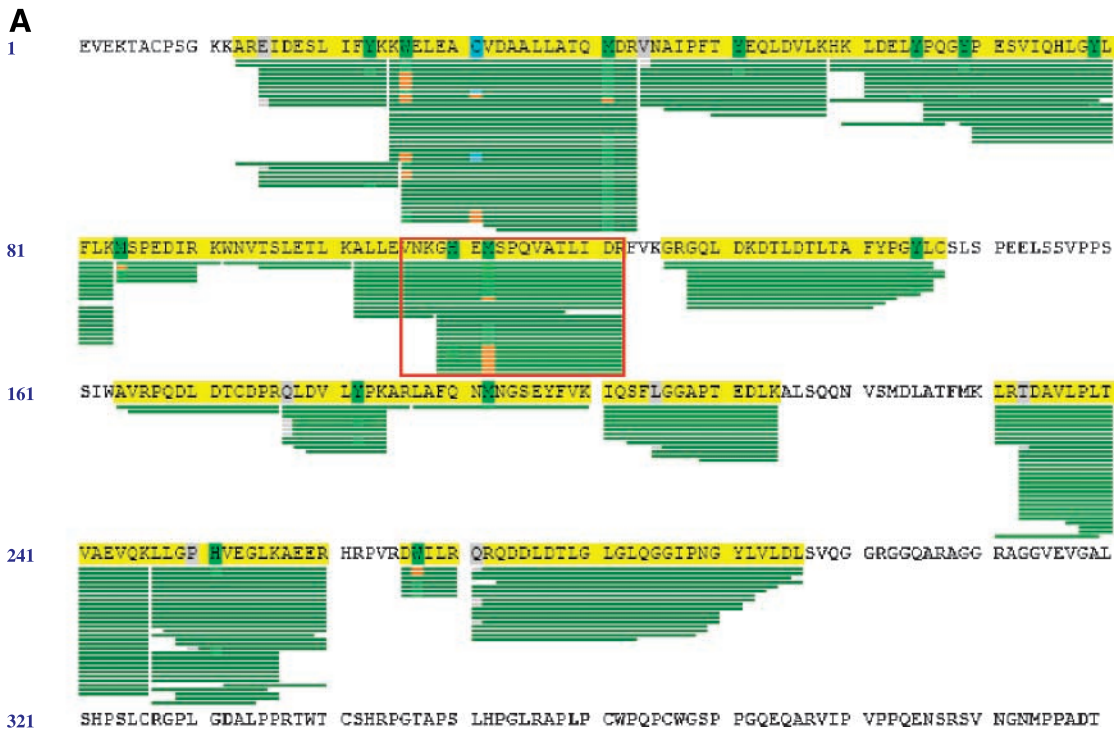


**Figure 3.** Ascites from an ovarian cancer patient was purified by immunoaffinity chromatography using mAb 569. Eluted proteins were separated by SDS-PAGE and a diffuse 40- to 44-kDa band was excised from the gel. The proteins were analyzed further by high-performance liquid chromatography-mass spectrometry. **A.** Alignment to the predicted sequence of variant 3. Recovered peptides mapped mostly to mesothelin variant 1 and variant 2, amino acids 13-297 (**B**). The COOH terminus was not resolved; thus, variant 3 could not be distinguished. *Yellow shading,* protein sequence covered by peptides; *dark green shading,* peptides detected; *light green shading,* MWHY* +15.995 oxidation (low pH artifact); *orange shading,* MWC^: +31.99 oxidation (low pH artifact); *gray shading,* nt] 17.05 water or ammonia loss (process artifact); *blue shading,* C# 71.015 propionamido-Cys (gel artifact).

**Antigen Expression at the Tumor Cell Surface according to Flow Cytometry.** Data summarizing assays on live cells are presented in Table 2.

According to these data, mAb 1A6-10, which is specific for variant 1, bound to live cells from ovarian carcinoma, mesothelioma, and pancreatic carcinoma. Similar results were obtained with mAb 4H3, which recognizes variants 1, 2, and 3, and mAb 569, which recognizes variants 1 and 3. In contrast, mAb 2B10, which is specific for variant 2, did not bind to ovarian carcinoma lines, although it bound to a fraction of cells from two of two mesotheliomas and two of three pancreatic carcinomas. mAb 8C8, which recognizes only variant 3, did not bind significantly to any of the tested cell lines. None of the mAbs bound to T51 cells used as negative control. We conclude that variant 1 is the biomarker more frequently expressed than variant 2 or 3 at the surface of cells from certain tumors, including ovarian carcinoma.

**Construction of a Standard Curve to Quantitatively Measure SMRP.** As shown in Fig. 4, the assay shows a high reproducibility with an imprecision of 6% to 15% coefficient of variance across the range of the assay. We calculated the limit of detection as the concentration corresponding to 2.5 times the SD of the background absorbance above the mean absorbance of the background. Based on averages obtained from multiple SMRP assays (data not shown), the limit of detection was determined to be 200 pg/mL with an average coefficient of variance of <15% at the limit of detection. Using the standard curve, it is possible to determine the antigen concentration in body fluids expressed in pg/mL.

**Table 2. Flow cytometry analysis of live carcinoma cells using mAbs to different mesothelin variants (as indicated within parentheses under each mAb)**

| Tumor | | mAb | | | | |
|---|---|---|---|---|---|---|
| Name | Type | 4H3 | 569 | 1A6-10 | 2B10 | 8C8 |
| | | (1, 2, and 3) | (1 and 3) | (1) | (2) | (3) |
| 1GROV1cp | Ovarian cancer | 89 | 80 | 89 | 0 | 0 |
| 1GROV1 | Ovarian cancer | 96 | 94 | 97 | 0 | 0 |
| 2008C13 | Ovarian cancer | 89 | 91 | 91 | 0 | 0 |
| HE50 | Ovarian cancer | 56 | 69 | 65 | 0 | 0 |
| Mesofr | Mesothelioma | 98 | 98 | 98 | 18 | 5 |
| He1MT | Mesothelioma | 83 | 81 | 92 | 13 | 0 |
| He1Pa | Pancreas cancer | 87 | 56 | 86 | 21 | 1 |
| He5Pa | Pancreas cancer | 71 | 72 | 73 | 0 | 0 |
| He7Pa | Pancreas cancer | 40 | 25 | 41 | 0 | 0 |
| T51 | Lymphoid cells | 2 | 3 | 4 | 2 | 0 |

NOTE: Data shown are percentage stained cells. The T51 B-lymphoid cell line was used as negative control.

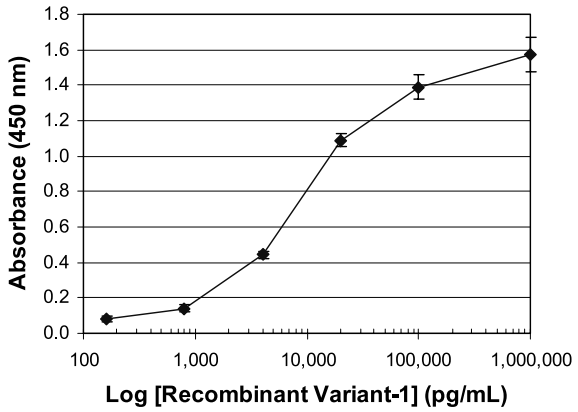


**Figure 4.** Standard curve of variant 1. ELISAs displaying the mean absorbance values from seven individual assays using mAbs 569 and 4H3 (10). *Points,* mean; *bars,* SD. Antibody 4H3 was coated on plates followed by a bovine serum albumin block. Subsequently, recombinant variant 1 fusion protein was applied in duplicate repeats on each plate and detected by biotinylated mAb 569. Bound mAb 569 was visualized by a streptavidin-horseradish peroxidase conjugate and a TMB substrate step. Mean absorbance values of each plate were plotted against concentrations of variant 1 fusion protein.

**Construction and Evaluation of an ELISA Specific for Variant 3.** Two mAbs which bound to variant 3 but not to variant 1 or 2 (Fig. 1), 4A10 and 8C8, were first used for a double determinant (sandwich) ELISA. The ELISA was constructed similar to the ''standard'' ELISA (10), detecting variants 1 and 3. Significant binding to variant 3 fusion protein was observed to a maximal concentration of 0.1563 μg/mL (data not shown). Detection of variant 3 was further improved by instead constructing an assay in which mAb 4A10 was replaced by mAb 4H3 (anti–variants 1, 2, and 3) and combined with mAb 8C8 (anti–variant 3). In parallel, we determined that mAb 4A11 (also specific for variant 3) was no better than mAb 4A10. The 4H3-8C8 assay was subsequently used to measure variant 3.

As shown in Fig. 5, studies on sera from 41 unselected patients, all of whom had clinical evidence of ovarian cancer (stages III or IV), showed that the 8C8/4H3 assay, detecting variant 3, identified much fewer positive sera (6 of 41) than the original ELISA (28 of 41), and the assay for variant 3 did not identify as positive any patient serum not also recognized by the original ELISA. Both assays had a similar level of specificity with <10% false-positive sera from patients with benign gynecologic disease. We conclude that variant 1 is a better biomarker than variant 3 for use in serum assays by having higher sensitivity. In view of the failure of the assay for variant 3 to detect most cases of advanced disease, no attempts were made to evaluate it for detection of early stage cancer.

## Discussion

It was previously reported that soluble mesothelin variant(s) of ~40,000 Da can be detected in supernatants of antigen-positive tumors as well as in sera and malignant effusions from patients with certain tumors [primarily carcinomas of the ovary (10, 12) and mesothelioma (11)]. Initial determination of the amino acid sequence for the soluble molecule(s) (10) showed identity with the $NH_2$-terminal end of mesothelin (4, 6, 7) and of a precursor of MPF (9). To further support this finding, two fusion proteins with human immunoglobulin were constructed, D1hIg and D2hIg, corresponding to the

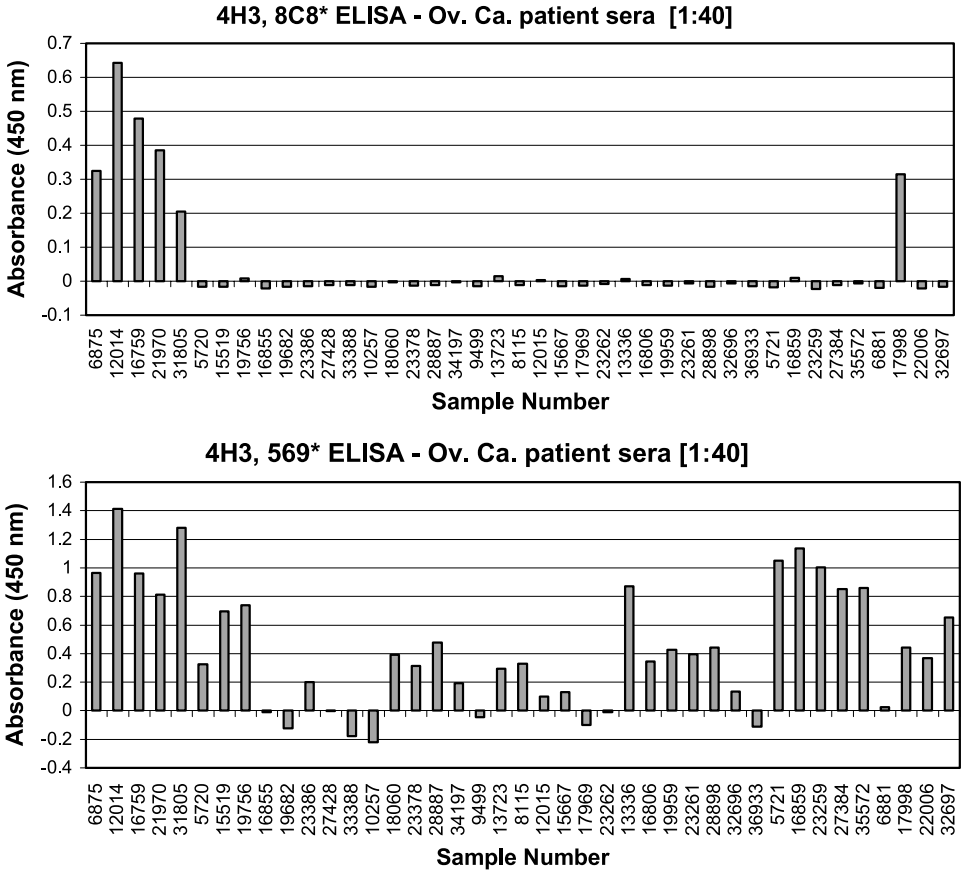


**Figure 5.** Results from testing of the same unselected sera from 41 patients with stage III or IV ovarian carcinoma with the ELISA specific for variant 3 (*top*) and with the standard ELISA detecting variants 1 and 3 (*bottom*). The absorbance values from the negative control were subtracted in both graphs. A positive sample was included for each assay.

published 33-kDa soluble domain of MPF (9) and to the published 44-kDa membrane-bound domain of mesothelin (6), respectively, which together correspond to the pre-pro-MPF. mAb 569 was found to bind to D2hIg but not to D1hIg (10). In view of previously published data from Pastan's group, according to which mesothelin is stably expressed at the tumor cell surface and not released into the circulation (4, 6, 7), and the finding that mAb 569 does not bind to D1hIg, Scholler et al. (10) speculated that the soluble molecule they had identified was the mesothelin variant (now known as variant 3) with an 82-bp insert.

Based on the published sequences for mesothelin variants 1, 2, and 3, we made fusion proteins with an Fc tail of mouse or human origin. The mouse-Fc tail fusion proteins were used to immunize mice and obtain mAbs to each mesothelin variant whereas the human-Fc tail fusion proteins were applied to analyze the new mAbs as well as the two mAbs, 569 and 4H3, which were used in a previously described and used sandwich ELISA (10-12). mAb 569 was found to bind to variants 1 and 3 and mAb 4H3 binds to all three variants (i.e., the published ELISA identifies variants 1 and 3). In contrast, mAb 1A6-10 is specific for variant 1, 2B10 is specific for variant 2, and 8C8 is specific for variant 3.

Although we had initially speculated that an assay that measures variant 3 may be advantageous, this was not the case, most likely because this variant, like variant 2, is much less frequently expressed at the surface of tumor cells according to flow cytometry. The prevalent expression of variant 1 is in agreement with analysis of the respective genes (13, 14).

We did not detect any molecules of ~69 kDa in culture supernatants or malignant effusions in our previous studies (10). In the present study, ascites from a patient with advanced ovarian carcinoma was analyzed. Western blotting only revealed bands of ~40 kDa. According to peptide analyses, both variant 1 and variant 2 could be isolated as soluble proteins from the ascites whereas the full-length precursor at 69 kDa was not detected. The possibility that such a molecule would occur in body fluids is unlikely because there is an Arg-Pro-Arg-Phe-Arg-Arg sequence between D1 and D2 in natural MPF (9), which can be processed by furin, a homologue of yeast precursor-processing endoprotease Kex2. Although the MPF precursor molecule can be made in COS cells and CHO cells transfected with the full-length gene, only the D1 part (~30 $K_d$) has been detected in culture supernatants. Most likely, the precursor protein is processed *in vivo*, preventing the detection of molecules with an approximate molecular weight of 70 kDa. Several polypeptide hormones are synthesized as large precursor proteins, such as human pro-parathyroid hormone–related protein (22), mouse pro-nerve growth factor (23), and human pro-platelet-derived growth factor (24). In each of these cases, active polypeptide sequences are usually bonded by pairs of basic amino acid residues such as Arg-Arg or Lys-Arg, and it is at these sites that proteolytic processing occurs. Watanabe et al. (25, 26) have shown that the sequence Arg-*X*-Arg/Lys-*X*-Lys/Arg-Arg is a signal for precursor cleavage catalyzed with furin. The sequence Arg-Pro-Arg-Phe-Arg-Arg deduced from the MPF cDNA matches this cleavage signal model. The COOH-terminal Arg residues of the 33- and 30-kDa rMPFs, Arg and Arg, are located 9 and 34 amino acid residues upstream of this cleavage signal, respectively. Moreover, the hydrophobicity profile predicts that the signal is located in a hydrophilic region between Trp and Glu. This suggests that the MPF precursor would fold and then be successively processed with furin-like and trypsin-like proteases. A similar processing has been found in leukocyte-derived natural IFN-α (27) and IFN-γ (28, 29).

We conclude that mesothelin variant 1, with an approximate molecular weight of 40 kDa, is released as a soluble molecule from certain tumor cells, including cells from most ovarian carcinomas and mesotheliomas, and that this is the biomarker previously shown to have clinical promise (10-12). We also conclude, as have others (13, 14), that mesothelin 1 is also more frequently expressed than variants 2 and 3 at the surface of cells from certain tumors as a potential therapeutic target. Studies on patients with early disease, as well as prospective studies on high-risk subjects, are needed to further establish its clinical value when used alone or in combination with other biomarkers such as CA125.

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