New Monoclonal Antibodies to Mesothelin Useful for Immunohistochemistry, Fluorescence-Activated Cell Sorting, Western Blotting, and ELISA

Masanori Onda,[1] Mark Willingham,[2] Satoshi Nagata,[1] Tapan K. Bera,[1] Richard Beers,[1] Mitchell Ho,[1] Raffit Hassan,[1] Robert J. Kreitman,[1] and Ira Pastan[1]

Abstract

Purpose: Mesothelin is a cell surface protein that is highly expressed in some malignant tumors, and is a promising target for immunotherapy. Recent data suggests that mesothelin is an adhesive protein and may have a role in the metastases of ovarian cancer. Although a few monoclonal antibodies (MAb) to mesothelin have been produced, they have limitations for the study of expression of native mesothelin because of their low affinity or reactivity only with denatured mesothelin protein. We have produced novel MAbs to mesothelin to help study mesothelin function and to develop improved diagnosis and immunotherapy of mesothelin-expressing tumors.

Experimental Design: Mesothelin-deficient mice were immunized with plasmid cDNA encoding mesothelin, and boosted with a mesothelin-rabbit IgG Fc fusion protein prior to cell fusion. Hybridomas were screened by an ELISA using plates coated with mesothelin-Fc protein.

Results: Seventeen hybridomas producing anti-mesothelin antibodies were established and shown to react with two epitopes on mesothelin. One group reacts with the same epitope as the low affinity antibody K1 that was originally used to identify mesothelin. The other is a new group that reacts with a new epitope. One antibody from each group was chosen for further study and shown to react strongly on ELISA, on immunohistochemistry, and by fluorescence-activated cell sorting on living cells.

Conclusion: Our two newly established MAbs, MN and MB, have different and useful properties compared with current antibodies used for the detection of mesothelin by immunohistochemistry, fluorescence-activated cell sorting, ELISA, and Western blotting.

Mesothelin is a 40-kDa glycosylphosphatidylinositol-linked cell surface glycoprotein present on normal mesothelial cells that is highly expressed in mesothelioma, ovarian cancer, pancreatic cancer, and some other malignancies (1–4). The normal biological function of mesothelin is unknown and mesothelin-deficient mice have no phenotype (5). However, a recent report indicates that mesothelin can bind to CA125/MUC16, suggesting that mesothelin might have a role in the metastatic spread of ovarian cancer (6). Initial studies of mesothelin expression were done by immunohistochemistry with monoclonal antibody (MAb) K1 generated by immunization of mice with the human ovarian carcinoma cell line OVCAR-3 (1, 2, 4). These studies showed that mesothelin is highly expressed in many human cancers including ovarian cancers, mesotheliomas, and squamous cell cancers. The studies were done on frozen sections, because MAb K1 did not work well on fixed tissues (7). However, using antigen retrieval by incubating tissue sections in 3 mol/L urea, mesothelin expression can be detected in paraffin-embedded formalin-fixed tissues. Subsequently, MAb 5B2 was generated by immunizing mice with a recombinant prokaryotic fusion protein corresponding to 100 amino acids which are present in the NH_2 terminus of mesothelin (8). Several immunohistochemical studies have been done with MAb 5B2 (9, 10). The studies confirmed that mesothelin is expressed in mesotheliomas, ovarian cancers, and squamous cell tumors and also showed expression in pancreatic cancer and lung cancer, although the distribution in lung cancer was diffuse and not limited to the plasma membrane. No expression of mesothelin was observed in lung cancer using MAb K1 (1). Because of its high expression in cancers and limited expression on normal tissues, mesothelin is also a promising target for cancer immunotherapy. Immunotoxins are chimeric proteins composed of the Fv portion of an antibody fused to a 38 kDa fragment of *Pseudomonas* exotoxin A (PE38). One of these, BL22, targets the cell surface protein, CD22, and has produced many complete remissions in drug-resistant Hairy Cell Leukemia showing that these agents can be useful in treating cancers in humans (11). We prepared immunotoxin SS1 (dsFv)-PE38 (SS1P) composed of an antibody fragment reacting with

Authors' Affiliations: [1]Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland and [2]Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina
Received 3/15/05; revised 5/10/05; accepted 5/26/05.
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doi:10.1158/1078-0432.CCR-05-0578

mesothelin and PE38 for the treatment of mesothelin-expressing cancers. SS1P has been shown to specifically kill mesothelin-expressing cell lines and to cause regressions of mesothelin-expressing tumors in mice (12, 13). Based on these studies and appropriate safety data, two phase 1 trials with SS1P are being conducted at the National Cancer Institute in patients with mesothelin-expressing cancers (14, 15). In addition, other therapies targeting mesothelin are in preclinical development (16).

Hellstrom and colleagues developed an ELISA-based assay to detect a protein in the blood they term soluble mesothelin–related protein (17). The exact relationship of this protein to mesothelin is currently not clear. Interestingly, soluble mesothelin–related protein is elevated in many patients with mesothelioma and some patients with ovarian cancer (18).

Research in the mesothelin area has been hampered by the lack of well-characterized, readily available antibodies that could be used for immunohistochemistry on fixed tissues, Western blotting, fluorescence-activated cell sorting (FACS) analysis of cells from patients, and ELISA to measure mesothelin in the blood and body fluids and other purposes. We describe here two new antibodies reacting with different epitopes on mesothelin that can be used for all these applications.

Materials and Methods

Generation of a mesothelin-Fc fusion protein by mammalian cells. The extracellular domain of the human mesothelin was expressed as a fusion protein with rabbit IgG Fc in HEK 293T cells. The DNA fragment encoding rabbit IgG Fc was amplified by PCR using the plasmid pγB1-12,14 for the RFc (amino acids 96-323, Swissprot, kindly provided by Dr. Rose G. Mage, NIH, ref. 19) as the template and inserted between *Sfi*I and *Sac*II sites of pSecTag2 (Invitrogen, Carlsbad, CA). cDNA for the extracellular domain of mesothelin was inserted between the *Sac*II and *Not*I to obtain the plasmid pOND-rFc-Meso. Primers used were as follows: Meso forward; 5′-AGA TAG AGT CCG CGG GGA GGT GAA GTG GAG AAG ACA GCC TGT-3′, Meso reverse; 5′-TTG TAT AGC GGC CGC TCA TCC CCC CGA GAG GGC CTC TTG CAC-3′. The plasmid was transfected into 293T cells by LipofectAMINE reagent (Invitrogen). The mesothelin-Fc protein harvested from the culture supernatant and purified with Hi-trap protein A column (Amersham Biosciences Corp., Piscataway, NJ). The purified proteins were quantitated by Coomasie blue (Pierce, Rockford, IL) and checked on SDS-PAGE gel.

Generation of a mesothelin protein by Escherichia coli. The mesothelin gene (GenBank accession number AY743922) was cloned from the IMAGE cDNA clone ID 5209096 using PCR and put it into pMAL-p2X (NEB, Beverly, MA) which has a tobacco etch virus protease cleavage site. The resulting plasmid pMH103 encodes a fusion protein consisting of the malE signal sequence, malE and mesothelin. The *malE* gene encodes the maltose-binding protein. The fusion protein is directed to the periplasm of *Escherichia coli*. The secreted proteins were separated with recombinant tobacco etch virus (Invitrogen). The samples were applied to an amylase column for removal of maltose-binding protein. The purity of bacterial mesothelin was >95%.

Cells. The pancreatic cancer cell line Panc 3.014 was obtained from Dr. Elizabeth Jaffee (Johns Hopkins Medical Institute, Baltimore, MD; ref. 16). It was maintained in RPMI 1640 supplemented with 20% fetal bovine serum, 200 μmol/L L-glutamine, 50 units/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 2 units/mL human insulin. The human mesothelioma cells NCI-H226 was obtained from Dr. Isaiah J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX). It was maintained in modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids. The human ovarian carcinoma cell line A1847 and human cervical carcinoma cell line HeLa were grown in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Immunization. Female mesothelin-deficient mice (6-8 weeks old) were immunized four times with the pcDNA3CAK1-9 plasmid intradermally (50 μg in 50 μL PBS) with 2-week intervals, and one boost was given with the mesothelin-Fc i.p. (50 μg in 100 μL of PBS) before fusion (5). Spleens were harvested 84 to 90 hours after the last boost for cell fusion. All animals were maintained in accordance with institutional guidelines.

Cell fusion. Cell fusions of the splenocytes and the SP2/0 cells were carried out according to the standard fusion protocol (20). Fourteen days after fusion, the supernatants were harvested and screened for antibody production by ELISA. The selected hybridomas were grown in a CELLINE flask (INTEGRA Biosciences, Chur, Switzerland) and purified on a protein A column (Amersham Biosciences).

Screening by ELISA on mesothelin-Fc fusion protein. Screening of hybridomas was done by ELISA. Briefly, Nunc-Immuno plates (Nalge Nunc International, Rochester, NY) were coated with 2 μg/mL of mesothelin-Fc protein. Plates were incubated overnight at 4°C. Then plates were blocked in blocking buffer (PBS with 25% DMEM, 5% FCS, 25 mmol/L HEPES, 0.5% bovine serum albumin, and 0.1% Azide) for 30 minutes at room temperature, washed twice with washing buffer (PBS with 0.05% Tween 20), and incubated with 100 μL supernatant of hybridoma for 1 hour at room temperature. After washing, plates were incubated with a horseradish peroxidase–conjugated goat anti-mouse antibody diluted 1:2,000 in blocking buffer. Finally, plates were washed with washing buffer and 100 μL immunopure tetramethylbenzidine substrate solution (Pierce) was added to each well. The color was allowed to develop for 2 to 5 minutes at room temperature and the reaction stopped by the addition of 50 μL 2 N solution of sulfuric acid. The plates were read at OD 450 nm using an automated plate reader (Molecular Devices Corp., Sunnyvale, CA).

Anti-mesothelin MAbs as control. MAb K1 was isolated and purified in our laboratory as previously described (2, 21). MAb 5B2 was obtained from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom).

Antibody quantification. For the determination of MAb concentration, Sandwich ELISA was used. Nunc-Immuno plates (Nalge Nunc International) were coated with 2 μg/mL of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., Grove, PA) in PBS overnight 4°C. One hundred microliters of blocking buffer was added to each well and then incubated for 30 minutes. After washing, serial diluted samples were added. As a standard immunoglobulin, mouse immunoglobulin classes and subclasses (Zymed Laboratories Inc., San Francisco, CA) were added. After washing, the bound MAbs were detected by a 1-hour incubation with horseradish peroxidase–labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) followed by tetramethylbenzidine substrate kit (Pierce). After adding 2 N-H_2SO_4 50 μL, the plates were read at OD_{450}. A standard curve was fit with the four-parameter logistic curve-fitting method.

Surface plasmon resonance assay. The affinity of the MAbs to mesothelin-rFc was determined by surface plasmon resonance using BIACore (Biacore, Piscataway, NJ). Three hundred and fifty response units of mesothelin-rFc was immobilized onto the biosensor chip, CM5 (using the BIACore amine coupling kit). On and off rates of MAbs were measured in PBS by injecting 25 μg/mL of each MAb over the chip surface for 5 minutes and then allowing the bound material to dissociate for 5 minutes by flowing only the buffer solution over the chip. Binding kinetics were analyzed using BIAevalutation 2.1 software (22).

Flow cytometry. Cultured cells (2×10^5) were dissociated with dissociation buffer (Sigma-Aldrich, St. Louis, MO). Each sample was washed twice in FACS buffer (PBS plus 5% fetal bovine serum, and 0.1% sodium azide). One hundred microliters of each hybridoma supernatant was added to the cells and incubated for 1 hour at 4°C. Cells were then washed twice with FACS buffer, resuspended in 100 μL of a secondary antibody (R-PE conjugated goat anti-mouse IgG diluted

1:100; Biosource, Camarillo, CA) and incubated for another hour at 4°C. Finally, cells were washed twice and analyzed on a FACSCalibur machine (Becton Dickinson, Franklin Drive, NJ) using the CellQuest software.

Isotyping. The isotype of selected MAbs was determined using a mouse immunoglobulin isotyping kit (Roche Applied Science, Indianapolis, IN).

Western blot. Reactivity of anti-mesothelin MAbs to SDS-denatured antigen was tested in a Western blot analysis. Mesothelin-Fc and CD25-Fc were separated on 4% to 20% SDS polyacrylamide gels (Bio-Rad, Hercules, CA) under reducing conditions. Proteins were transferred to a 0.2 μm Immun-blot polyvinylidene difluoride membrane (Bio-Rad) in transfer buffer [25 mmol/L Tris-HCl, 192 mmol/L glycine, 30% (v/v) methanol (pH 8.3)] at 4°C for 1 hour at 240 mA. After blocking with blocking solution (Roche Applied Science), the membrane was incubated with 1 μg/mL of each MAb for 1 hour at room temperature. The bound MAbs were detected with alkaline phosphatase–labeled goat anti-mouse IgG (Biosource) and BDIP/nitroblue tetrazolium substrate (Pierce).

Immunohistochemistry. Archival paraffin-embedded formaldehyde-fixed tissue sections from patients with mesothelioma were evaluated for mesothelin expression (15). The sections were deparaffinized in xylene, followed by graded ethanol hydration into water. The sections were treated for antigen-retrieval either using: (*a*) a commonly used commercial antigen unmasking solution (Vector, Burlingame, CA); (*b*) citraconic anhydride in a boiling water bath at 97°C for 60 minutes (23); or (*c*) treatment with 3 mol/L urea in a boiling water bath for 60 minutes. These treatments were followed by blocking with 1% bovine serum albumin in PBS for 30 minutes at 23°C, then incubation with primary antibodies in 1% bovine serum albumin–PBS for 60 minutes at room temperature. Primary mouse antibodies 5B2 (Novocastra Laboratories), MAb K1, MAb MB, or MAb MN were used at (or at a preparation dilution equivalent to) 10 μg/mL. After washing in PBS, the sections were incubated with affinity-purified goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch) at 25 μg/mL in 1% bovine serum albumin–PBS for 30 minutes, followed by detection of peroxidase with diaminobenzidine-peroxide substrate solution for 10 minutes at 23°C. The sections were counterstained with hematoxylin.

Results

Generation of a recombinant mesothelin-Fc fusion protein and generation of MAbs. To obtain MAbs that recognize the extracellular domain of mesothelin, we used a DNA immunization protocol followed by protein immunization for the final boost prior to cell fusion (24). The mesothelin-Fc protein used for immunization and screening was prepared from the medium of transfected HEK293T cells by purification on protein A-Sepharose (22). Six mesothelin-deficient mice were injected intradermally with DNA prepared from the plasmid, pcDNA3CAK1-9. Blood was collected from the mice after multiple injections and the antibody titer was determined by ELISA on plates coated with mesothelin-Fc protein (data not shown). The two mice with the highest titer (>10^5) were sacrificed. Spleen cells from each mouse were fused to myeloma cells following standard procedures (20). Supernatants from clones of hybridoma cells were screened using ELISA. To determine the specificity of the antibodies, the screening was done on plates coated with mesothelin-Fc and CD25-Fc. The latter is a negative control that excludes antibodies reacting with the Fc portion of the fusion. Finally, 17 hybridomas were identified that reacted selectively with mesothelin-Fc and not with CD25-Fc. The topographical epitopes of these MAbs were identified based on the mutual competition of all possible pairs of the MAbs (25). Two topographical epitope groups were identified. One group had 2 clones and contained MAb MB, whereas the other group had 15 clones and contained MAb MN, which competes with and belongs to the same epitope group as MAb K1. The characteristics of the two new MAbs and the two standard anti-mesothelin MAbs, K1 and 5B2, are shown in Table 1. The affinities of the new anti-mesothelin MAbs determined by BIACore (K_ds of 1.0 and 0.6 nmol/L) are much higher than previous MAbs. Because the new MAbs have high affinities for native mesothelin in the BIACore format, we examined their performance in other types of assays. In these studies, we compared them with the two commercially available antibodies and found them to be useful in all the assays for mesothelin, whereas the two others did not detect mesothelin in all assay conditions. The data is shown below and summarized in Table 1.

Flow cytometry analysis of the anti-mesothelin supernatants. The 17 supernatants were tested by FACS for their

Table 1. Characterization of anti-mesothelin MAbs

MAbs	Isotype*	Affinity† (nmol/L)	FACS‡	ELISA§ (ng/mL)	Western blot‖ (ng)	Immunohistochemistry¶	Epitope**
MN	IgG_{2a}	1.0	3.5	1.1	2	+	1
MB	IgG_{2a}	0.6	3.3	1.9	2	+	2
K1	IgG_1	12	2.5	1,000	25	+	1
5B2	IgG_1	NA††	1.3	1,000	25	+	NA

*All MAbs contained a κ light chain.
† Affinity to mesothelin-Fc in solution determined by BIACore.
‡ Reactivity to H226 cells in FACS (log geometric mean of fluorescence intensity). Each MAb (1 μg/mL) was incubated with H226 (mesothelin-positive) cells and the bound MAb was detected by PE-labeled anti-mouse IgG. FACS histograms are shown in Fig 1. The values are geometric means of FACS signals. All the anti-mesothelin MAbs reacted to H226 cells.
§Reactivity to ELISA. ELISA plates were coated with mesothelin-Fc. After incubation with each MAbs, the bound MAb was detected by HRP-labeled anti-mouse IgG. The values are the amounts of MAbs that showed OD_{450} = 0.5.
‖Reactivity to SDS-denatured mesothelin-Fc in Western blot (Fig. 3). The values are the minimum amount of mesothelin-Fc from which each MAb can be detected with.
¶ The results of K1 and 5B2 were cited from previous reports (2, 8, 9).
**Topological group of epitopes identified based on the mutual competition of the MAbs (24).
†† Not applicable because 5B2 does not bind well to mesothelin-Fc.

ability to bind to the Panc 3.014 mesothelin-expressing cell line. All 17 antibodies bound to Panc 3.014 cells but did not bind to the A431 mesothelin negative cells (data not shown). One antibody from each epitope group, MN and MB, was selected for further study based on the highest signal on FACS. Representative FACS analysis of the new antibodies MN and MB, as well as MAb K1 and MAb 5B2 on H226 lung cancer cells, A1847 ovarian cancer cells, HeLa cervical cancer cells, and Panc 3.014 pancreatic cancer cells are shown in Fig. 1. The data in Fig. 1 show that MAbs MN and MB generated a large increase in fluorescence intensity compared with the cells incubated with the control anti-CD30 antibody. The signal was highest on H226 and Panc 3.014 cells followed by A1847 and HeLa cells. MAb K1 also reacted with H226, Panc 3.014, and A1847 cells but the signal was weaker than with MAbs MN or MB. Using MAb 5B2, the fluorescence signal was weak on H226 and negative on A1847, HeLa, and Panc 3.014 cells. With all antibodies, no reactivity was detectable in mesothelin-negative cells, A431 (data not shown). These data show that MAbs MN and MB are superior to the other MAbs for FACS analysis. The isotype of MAbs MN and MB are IgG_{2a}.

ELISA. To assess the reactivity of the MAbs in an ELISA format, plates coated with 2 μg/mL of mesothelin-Fc were exposed to increasing concentrations of each of the MAbs. Figure 2A shows representative data from triplicate experiments. MAb MN showed the strongest signal followed by MAb MB. MAbs K1 and 5B2 showed a very weak signal. In a control ELISA using plates coated with CD25-Fc, none of the antibodies showed a signal (data not shown). Thus, MAb MN seems to be the best choice for detection of mesothelin in this ELISA format. In another ELISA format, plates were coated with mesothelin produced in *E. coli* and the reactivity of the antibodies compared. Figure 2B shows representative data from triplicate experiments. MN showed a very strong signal followed by 5B2, which showed a weak signal; K1 and MB, showed a very weak signal.

Western blot. The reactivity of the anti-mesothelin MAbs to SDS-denatured antigen was tested in a Western blot analysis in which various amounts of mesothelin-Fc were loaded on a gel and blotted with each antibody. Figure 3 shows that MAbs MN and MB are 10 times more sensitive in detecting mesothelin on the Western blot than MAbs K1 and 5B2. For specificity controls, we showed that none of the antibodies reacted with the CD25-Fc control protein. In addition, monoclonal anti-CD30 antibodies did not react with mesothelin-Fc or CD25-Fc protein. This result suggests that these MAbs probably recognize a linear epitope on mesothelin.

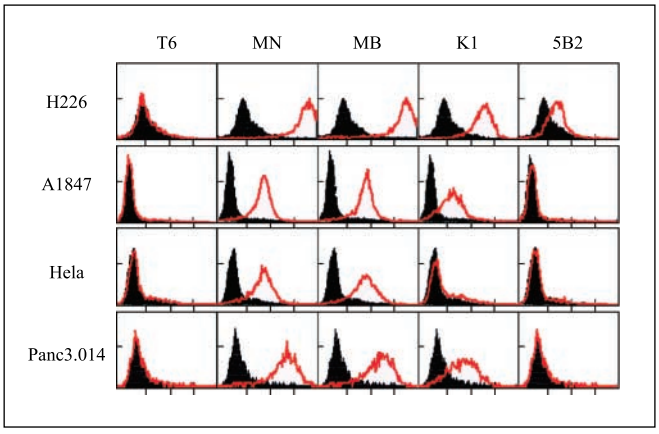

Fig. 1. FACS analysis of anti-mesothelin MAbs with different cells. Cells were incubated with the anti-mesothelin MAbs (1 μg/mL) or anti-CD30 MAbs,T6, as a negative control, followed by PE-labeled goat anti-mouse IgG. Each histogram shows the staining with first antibody (red line) and without first antibody (gray shadow). First antibody and cells used are shown. Mean fluorescence intensities for Table 1 were measured under the same conditions for all experiments.

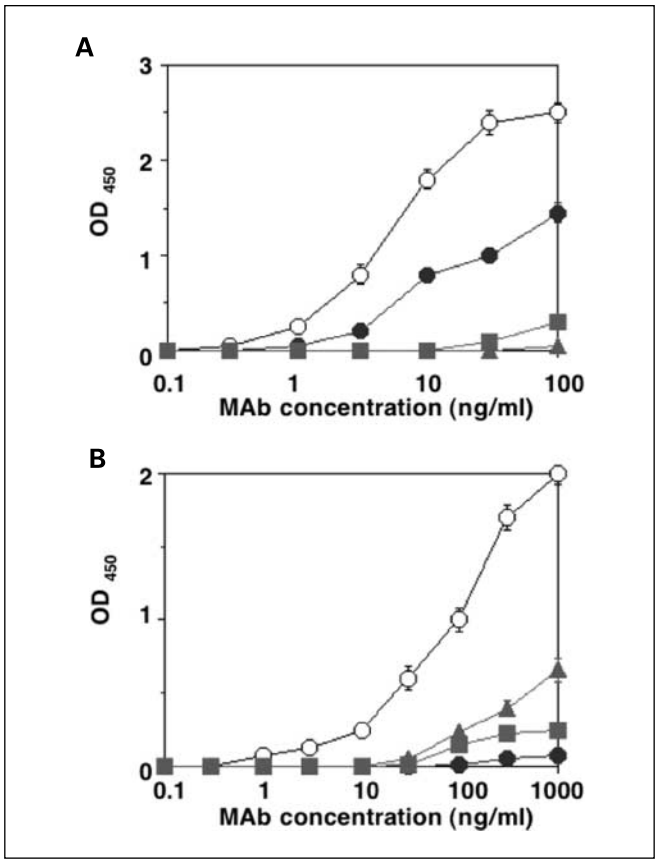

Fig. 2. MAb reactivity on ELISA coated with mesothelin-Fc (*A*), or bacterial maltose-binding protein – mesothelin (*B*). ELISA plates were coated with mesothelin-Fc (2 μg/mL) or bacterial mesothelin (2 μg/mL). Then, ELISA plates were incubated with anti-mesothelin MAbs, MN (○), MB (●), K1 (■), or 5B2 (▲), followed by horseradish peroxidase – conjugated goat anti-mouse IgG. Experiments were done in triplicate. Points, mean; bars, ± SD ($n = 3$).

Immunohistochemistry. The patterns seen using peroxidase immunohistochemistry with different anti-mesothelin antibodies in the same area of the same case of mesothelioma are shown in Fig. 4. In (*A*), sections treated with a commercial antigen retrieval reagent (Vector) show very poor reaction with a case of mesothelioma using MAb K1, whereas (*A′*) shows the result following antigen retrieval using 3 mol/L urea. The levels of reactivities detected under these conditions are comparable to those seen in the detection of mesothelin using MAb K1 in frozen sections (data not shown). Thus, MAb K1 can detect mesothelin expression, but only in frozen sections or paraffin sections treated using aggressive antigen retrieval, and is much less effective using more common commercial antigen retrieval reagents. As described below, a new aggressive antigen retrieval method using citraconic anhydride also improved K1 reactivity. On the other hand, MAb 5B2, a commercial antibody from Novocastra Laboratories, shows good reactivity after commercial antigen retrieval (*B*), a level of intensity similar to MAb MN

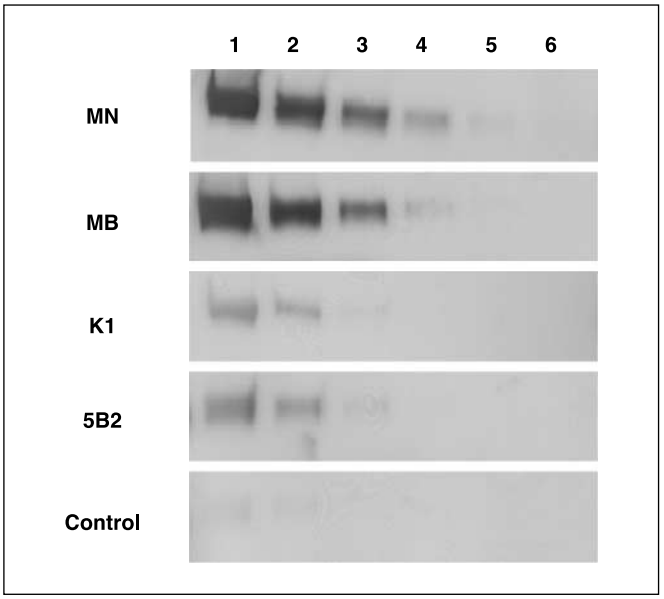

Fig. 3. Western blot analysis of anti-mesothelin MAbs. Mesothelin-Fc (lane 1, 100 ng; lane 2, 25 ng; lane 3, 6 ng; lane 4, 2 ng; and lane 5, 0.4 ng) and CD25-Fc (lane 6, 50 ng) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with each anti-mesothelin MAbs (1 μg/mL) or anti-CD30 MAb, T6 (control), followed by ALP-goat anti-mouse IgG and BCIP/nitroblue tetrazolium substrate. Each experiment was done under the same conditions. Data are summarized in Table 1.

shown in (*D*). MAb MB, however, shows superior reactivity with commercial antigen retrieval, as shown in (*C*). This pattern was observed in three separate cases of mesothelioma examined using these reagents. This suggests that MAb MB may be a superior reagent for use with commonly used commercial immunohistochemical procedures in archival paraffin-embedded tissues. We also showed that our newly established MAbs are specific for mesothelin-expressing cells using tissue arrays with different cancers and normal controls (Fig. 5; Table 2). These latter comparisons were made using citraconic anhydride antigen retrieval, a recently introduced reagent for antigen retrieval that is effective for epitopes that are difficult to unmask, including the K1-reactive epitope (ref. 23; data not shown).

Discussion

We have succeeded in producing useful antibodies to the membrane protein mesothelin by DNA immunizations followed by a single boost of a recombinant mesothelin-Fc fusion protein produced by human cells. The newly established MAbs react strongly and specifically to two different epitopes on the native form of the mesothelin as well as with denatured mesothelin. These data confirm previous results with other proteins that DNA immunization followed by boost with a recombinant protein is an easy and useful method to produce MAbs against cell surface antigens.

For immunization, we used mesothelin-deficient mice, because they should not be tolerant to epitopes on human mesothelin that are also present in mouse mesothelin and therefore should react to more epitopes on mesothelin than normal mice (5). Using a mutual competition assay, we evaluated the epitopes recognized by the 17 new clones as well as those recognized by MAb K1 and immunotoxin SS1P (data not shown). We found that we obtained antibodies against only two epitopes. One of these epitopes was recognized by the K1 antibody previously made in normal mice. MAbs MN, K1 and immunotoxin SS1P, in which the Fv was obtained from an antibody phage library are in the same epitope group. MAb K1 was generated by immunization with the human ovarian cancer cell line OVCAR-3 (1, 2). On the other hand, the Fv of SS1P was cloned from the splenic mRNA of mice using antibody phage display. The mice were immunized with an expression vector coding for mesothelin.

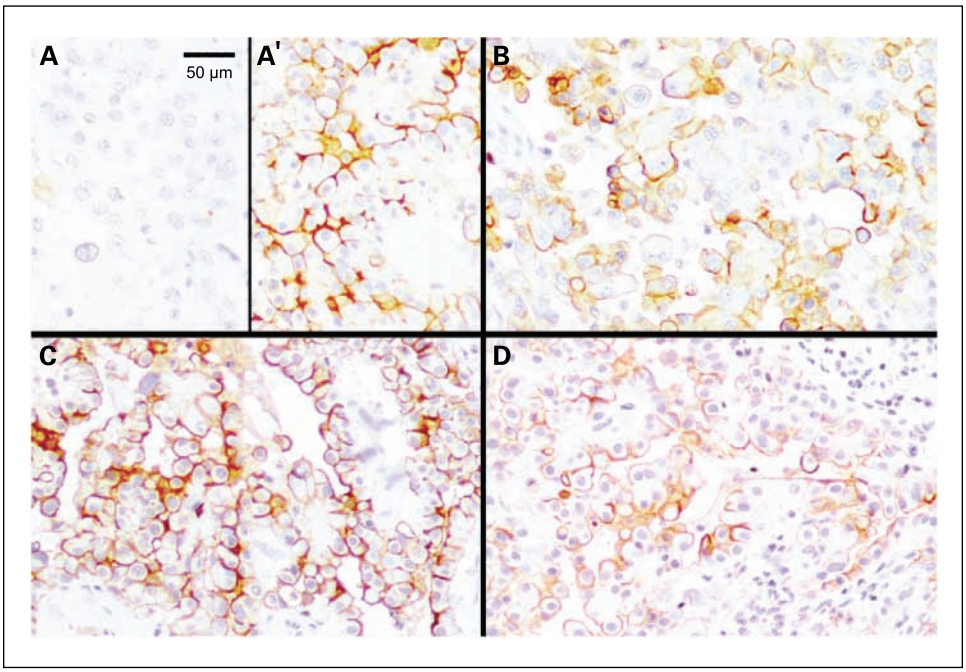

Fig. 4. Immunohistochemistry of anti-mesothelin MAbs. Peroxidase immunohistochemical results are shown from the same area of the same case of mesothelioma. Formaldehyde-fixed, paraffin-embedded tissue sections were treated with a commercial antigen retrieval procedure (Vector; *A-D*), but one section was treated with the 3 mol/L urea antigen retrieval (*A'*). Anti-mesothelin primary antibodies were used to label these sections as follows: A and A', MAb K1; B, MAb 5B2; C, MAb MB; and D, MAb MN. Note that MAb K1 labels poorly with commercial antigen retrieval, but is much stronger using the 3 mol/L urea treatment, whereas the other antibodies label well using commercial antigen retrieval, with MAb MB (*C*) showing the most intense reaction (*magnification,* ×200; *bar,* 50 μm).

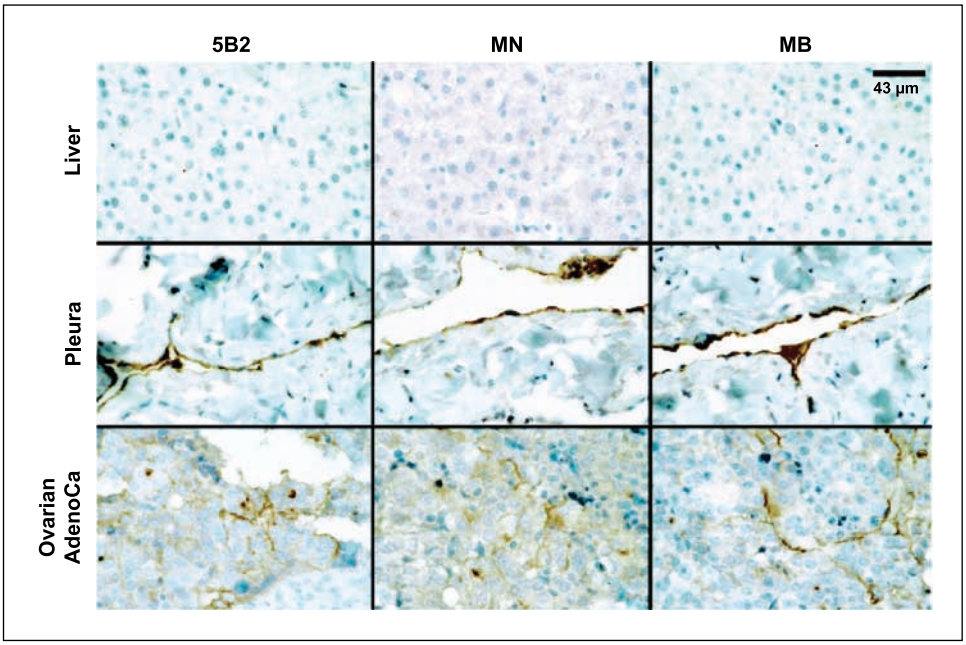

Fig. 5. Immunohistochemistry of anti-mesothelin MAbs on tissue arrays. Representative peroxidase immunohistochemical results are shown. Paraffin-embedded tissue sections (as shown in Table 2) were obtained from Biochain Institute (Hayward, CA) and treated with a citraconic anhydride antigen retrieval procedure (23). The complete results including other tissues are summarized in Table 2 (*bar,* 43 μm).

Fifteen of the 17 newly established MAbs reacted with this epitope. Because such antibodies are frequently generated by three different methods of immunization, this must be a dominant epitope (1, 11). MAb MN reacts with mesothelin-Fc protein and with mesothelin made in *E. coli*, showing that this epitope is present on recombinant mesothelin made in mammalian cells and in bacteria (Fig. 2). The epitope recognized by MAb MB does not react with mesothelin made in bacteria (Fig. 2B). MAb MB works very well for immunohistochemistry using a commercial antigen retrieval reagent (Vector) and should therefore be useful for such studies. On the other hand, even though it works for immunohistochemistry with commercial antigen retrieval methods, 5B2 reacts with the bacterial form of mesothelin, but not with the native mesothelin made in human cells.

The affinities of the new anti-mesothelin MAbs were measured by BIACore and found to have high affinities with K_ds of 1.0 and 0.6 nmol/L. These antibodies showed good performance when used for FACS analysis, ELISA, Western blot, and immunohistochemistry (Table 1). Thus, these MAbs are useful for detecting the mesothelin protein using all types of immunologic assays. Several studies have described the usefulness of detecting tumor mesothelin expression by immunohistochemistry as an aid in the pathologic diagnosis of mesothelioma, ovarian cancer, and pancreatic cancer. Although initial studies using MAb K1 on frozen sections suggested that mesothelin could help distinguish between mesothelioma and adenocarcinoma, a recent study using MAb 5B2 noted diffuse mesothelin expression in some lung adenocarcinomas (1, 10). Because mesothelin immunostaining is a very sensitive marker for epithelial mesothelioma, a negative mesothelin immunostain suggests a diagnosis other than mesothelioma (9). Tumor mesothelin expression has also been shown to be useful for the diagnosis of ovarian cancer. A study that looked at mesothelin expression by a variety of different tumors showed strong mesothelin expression by nonmucinous ovarian cancers but not in a variety of tumors with which these lesions may be confused; therefore, mesothelin staining can assist in establishing the differential diagnosis (10). Mesothelin expression in the majority of pancreatic ductal adenocarcinomas makes it a valuable tool

Table 2. Summary of immunohistochemical study on the tissue arrays

	5B2	MB	MN
Tumor			
Ovarian adenocarcinoma 1	+	+	+
Ovarian adenocarcinoma 2	focal+	focal+	focal+
Teratoma	–	–	–
Hepatoma	–	–	–
Breast ductal adenocarcinoma	–	–	–
Leiomyoma	–	–	–
Squamous cell lung carcinoma	–	–	–
Astrocytoma	–	–	–
Renal cell carcinoma	–	–	–
Normal tissues			
Peritoneal mesothelium (on uterus)	+	+	+
Pleural mesothelium (on lung)	+	+	+
Brain	–	–	–
Breast	–	–	–
Kidney	–	–	–
Fallopian tube epithelial cells	–	–	–
Fallopian tube luminal fluid	+	+	+
Liver	–	–	–
Lung	–	–	–
Uterus (endometrium, myometrium)	–	–	–
Ovarian stroma 1	–	–	–
Ovarian stroma 2	–	–	–
Placenta	–	–	–

NOTE: +, stained; –, not stained.

for diagnosis of these tumors (3). In a study by Hornick et al., mesothelin in combination with other tumor markers was useful in distinguishing metastatic pancreatic adenocarcinoma from bile duct adenomas and hamartomas of the liver that are at times difficult to diagnose by light microscopy alone (26). Also, mesothelin immunostaining was shown to be valuable in distinguishing metastatic pancreatic mucinous adenocarcinomas in the ovaries from primary ovarian mucinous neoplasm; this is critical for proper patient management (27). Mesothelin staining can also help in the interpretation of pancreatic fine-needle aspiration given the cytologic overlap of neoplastic and reactive processes (28).

Hellstrom et al. have identified a soluble member of the mesothelin/MPF family, called soluble mesothelin–related protein that is elevated in the serum of the majority of patients with ovarian cancer (17). A recent study that evaluated serum CA 125 and soluble mesothelin–related protein in ovarian cancer patients and normal risk controls showed that using both markers together may be superior to CA 125 alone for ovarian cancer screening and diagnosis (29). In addition, soluble mesothelin–related protein has been shown to be a useful marker for the diagnosis of pleural mesothelioma and to follow disease progression (18). We have evidence that a small amount of cell-bound mesothelin is shed into the serum; this could be a valuable test for the diagnosis and follow-up of patients with mesothelin-expressing cancers.[3]

The detection of mesothelin expression by immunohistochemistry, besides being useful to the pathologists in making a diagnosis, is also important clinically to select patients for therapies targeting mesothelin (8) and the development of assays to measure serum mesothelin would be useful for the diagnosis and follow-up of patients with mesothelin-expressing cancers.

In conclusion, we have established new MAbs that react with mesothelin. Two of these, MN and MB, have better overall performance characteristics than previous MAbs and should be useful in studying the biology of mesothelin and also aid in the diagnosis and therapy of mesothelin-expressing cancers.

[3] Our unpublished data.

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Clin Cancer Res 2005;11:5840-5846.