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Thrombomodulin Expression in Malignant Pleural Mesothelioma and Pulmonary Adenocarcinoma

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Thrombomodulin (TM) is a glycoprotein of molecular weight 75,000 kd that is normally present in restricted numbers of cells, including endothelial and mesothelial cells. In this study, the authors tested the possibility of using anti-TM to facilitate the diagnosis of mesothelioma. All of the 31 mesotheliomas and the two mesothelioma cell lines (MS-1 and MS-2) tested were stained positively with anti-TM. The specificity of anti-TM staining in mesothelioma cells was further confirmed by in situ *hybridization of MS-1 cells with a TM-specific probe. The expression of TM in MS-1 cells was increased markedly when these cells were induced by 12-0-tetradecanyl phorbol 13-acetate (TPA) to differentiate. The expression of TM in mesothelioma cells, however, did not correlate with any particular phase of the cell cycle. In an attempt to differentiate pleural mesothelioma from pulmonary adenocarcinoma, the authors compared the expression of TM, carcinoembryonic antigen (CEA), and Leu M1 in these two types of tumors. Only four of 48 (8%) pulmonary adenocarcinomas were stained positively by antibodies to TM. Therefore, immunohistochemical staining with antibodies to TM yielded 100% sensitivity and 92% specificity for diagnosis of mesothelioma. All of the mesotheliomas stained negatively for CEA and Leu M1, except for one, which showed minimal focal positivity for Leu M1. In contrast, 79% and 60% of adenocarcinomas stained positively for CEA and Leu M1, respectively. These findings suggest that immunocytochemical staining with anti-TM should be added to the battery of tests to increase the diagnostic sensitivity and specificity for differentiating mesothelioma from pulmonary adenocarcinoma. (Am J Pathol 1992, 141:827–833)*

The diagnosis of malignant pleural mesothelioma (MS), and in particular its distinction from adenocarcinoma of the lungs and other organs that result in metastases to the pleura or the pericardium, continues to pose difficult enigmas for the pathologist. It is important to distinguish between MS and other tumors because of differences in biologic behavior, treatment, and patient survival rates. Many methods have been used for making this distinction, including histochemical, electron-microscopic, flow-cytometric, and immunohistochemical analyses.[1–5] Histochemical stains such as mucicarmine, the periodic acid-Schiff method after diastase digestion, and the alcian blue method with or without hyaluronidase treatment are often used as screening tests. They are often inconclusive, however. The immunohistochemical method currently used employs antibodies to cytokeratins, vimentin, carcinoembryonic antigen (CEA), Leu M1 (CD15), epithelial membrane antigen, blood-group–related antigens, and antibodies to several glycoproteins (B72.3, 44-3A6, and 624A12).[6–8] A combination of these antibodies as well as the use of histochemical staining and electron-microscopic studies could improve the sensitivity and specificity for the differential diagnosis of mesothelioma and adenocarcinomas.

In an effort to increase the sensitivity and specificity for the diagnosis of mesothelioma, we examined the possibility of using an anti-thrombomodulin (TM) antibody. Thrombomodulin is a protein composed of 575 amino acids (molecular weight 75 kd),[9] which is normally expressed by a restricted number of cells, such as endothelial cells and mesothelial cells, but not by normal bronchial epithelium or other types of cells in the lungs.[10] Our

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purpose in this study was to demonstrate the expression of TM in mesotheliomas, and to use this characteristic as a means of differentiating between malignant pleural mesothelioma and primary pulmonary adenocarcinoma.

Materials and Methods

Diagnosis and Tissue Processing

Cells from 31 malignant pleural mesotheliomas (29 epithelial and two biphasic types), two human mesothelioma cell lines (MS-1 and MS-2),[11] and 48 primary pulmonary adenocarcinomas were studied. All of the material except for the cell lines was obtained from surgical specimens that had been fixed in buffered formalin and embedded in paraffin blocks. The diagnostic criteria used for adenocarcinoma were typical histology on hematoxylin and eosin (H&E)-stained sections, as well as positive staining with mucicarmine, CEA, or Leu M1.[8] The diagnostic criteria used for mesothelioma were typical histology on H&E-stained sections, negative staining with mucicarmine and CEA, and the presence of long, slender microvilli on electron microscopy. The diagnosis of mesothelioma was confirmed by histochemistry and electron microscopy in all cases.

Immunocytochemistry

We examined the expression of TM, Leu M1, and CEA in mesotheliomas and adenocarcinomas. A mouse monoclonal antibody (MAb) directed against a pure fragment of human TM was prepared by J. Morser and C. Edmon and was a gift from Berlex Biosciences (San Francisco, CA). The MAb anti-Leu M1 was obtained from Becton-Dickinson Laboratories (Sunnyvale, CA), and rabbit anti-human CEA was purchased from Dako Co. (Santa Barbara, CA). The labeling reagents biotin-conjugated horse anti-mouse IgG or goat anti-rabbit IgG and avidin-biotin-peroxidase complex (ABC) were obtained from Vector Laboratories (Burlingame, CA).

The staining procedure used has been described elsewhere in detail.[11] Briefly, the tissue sections were deparaffinized and rehydrated according to routine procedures. The sections were treated with MAbs at 2 μg/ml for 1 hour. After extensive washing, sections were sequentially incubated with biotin-labeled secondary antibody (1:400) and ABC and then developed in a substrate containing diaminobenzidine, nickel chloride, and hydrogen peroxide (DAB-Ni-H_2O_2), which gave a bluish-black reaction product. The sections were counterstained with methyl green, dehydrated, and cleared as in routine processing. Endothelial cells of blood vessels served as a positive internal control for staining with antibodies to TM. Pulmonary adenocarcinomas served as a positive control for staining with antibodies to Leu M1 and CEA. As a control for staining specificity, we replaced monoclonal anti-TM with mouse nonimmune ascites at equivalent concentrations.

Expression of TM in Normal and in Cultured Mesothelial Cells

Pleural fluid from noncancer patients was the source of normal and reactive mesothelial cells. A mononuclear cell fraction was prepared after gradient centrifugation. The presence of mesothelial cells was confirmed by coexpression of cytokeratin and vimentin in these cells. We also used two mesothelioma cell lines (MS-1 and MS-2) to study the expression of TM. These cells were cultured at 4×10^5 cells/ml in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mmol/l glutamine, 50 μmol/l 2-mercaptoethanol, and 50 μg/ml gentamicin at 37°C in a humidified, 5% CO_2 atmosphere. The medium was changed every 2 to 3 days. For immunostaining, cytospin smears of normal and reactive mesothelial cells and cultured MS cells were fixed in 4% paraformaldehyde at room temperature for 5 minutes. After being washed in TRIS-buffered saline (TBS), 0.01 mol/l (molar), pH 7.6, the smears were immunostained with anti-TM as described above.

Expression of TM in Relation to Cell Cycle

To examine whether the expression of TM is related to the cell cycle of MS cells, we performed double staining and examined the expression of TM and Ki-67 (Dako, Santa Barbara, CA). The staining was done on either 12-0-tetradecanyl phorbol 13-acetate (TPA)-treated or untreated MS-1 cells. Treatment with TPA had been shown previously to prevent cell exit from G_0 phase and entry into G_1 phase, but TPA does not interfere with entry of cells in G_1 and S phases into G_2 and M phases. The TPA induction (2 ng/ml) was carried out for 2 days, and the treatment protocol was the same as described previously.[11] We also carried out a DNA cell cycle analysis of MS-1 cells by using flow cytometry to confirm the effectiveness of TPA induction.

For double-immunostaining, cytospin smears of MS-1 cells were fixed in 4% paraformaldehyde for 5 minutes. After being washed in buffer, the smears were immunostained with anti-Ki-67, and the peroxidase reaction was developed with DAB-Ni solution. The smears were then washed and immunostained with anti-TM as described above, except that a different substrate (3-amino-9-

ethylcarbazole, AEC) was used, which gave a reddish-brown precipitate.

In Situ *Hybridization*

Probes

We prepared anti-sense and sense oligonucleotide probes according to bases 629-600 of the DNA sequence previously described for human TM. The relative positions and sequence of these probes are shown in Figure 1. These probes were synthesized by the solid-phase β-cyanoethyl phosphoramidite method on an automated DNA synthesizer (Cyclone Plus, MilliGen/Biosearch, Novato, CA) and were purified by electrophoresis on polyacrylamide gels.

Labeling

The synthetic oligonucleotide probes were labeled with biotin by incorporation of biotin-11-dUTP (Enzo Diagnostics, Inc., New York, NY) according to a modification of a terminal deoxyribonucleotide transferase (TdT) procedure described by the laboratories of British Biotechnology Limited (Abingdon, Oxon, UK). The assay solution contained 1 μl probe (100 pmoles), 8 μl 5 × TdT buffer (Gibo-BRL, Gaithersburg, MD), 15.0 μl biotin-11-dUTP (0.3 mmol/l), 12.5 μl diethylpyrocarbonate-treated water, and 3.5 μl TdT (15 units/μl). The reaction was carried out at 37°C for 3 hours. The probe was diluted in 1 mmol/l ethylenediaminetetra-acetic acid (EDTA), 10 mmol/l TRIS (pH 8.0), and stored at −20°C until use.

Hybridization

Mesothelioma cells were grown as a monolayer on Falcon-brand cell culture flasks (Becton Dickinson, Lincoln Park, NJ) at 37°C, 5% CO_2 in RPMI medium with 10% defined fetal calf serum (Hyclone Laboratories, Inc., Logan, UT). After a rinse in phosphate-buffered saline (PBS), the cells were fixed in 4% paraformaldehyde for 15 minutes, followed by three rinses in 70% ethanol. A PBS rinse was then repeated for 10 minutes, followed by a 10-minute rinse with 0.5 × SSC (0.03 mol/l citric acid trisodium and 0.3 mol/l sodium chloride). The slides were then immersed in 0.25% (vol/vol) acetic anhydride in 0.1 mol/l triethanolamine for 5 minutes and rinsed once in 2 × SSC for 10 minutes. A prehybridization solution consisting of 50% deionized formamide, 10% dextran sulfate, 2 × SSC, 0.12 mol/l EDTA, and 0.33 μg/ml salmon sperm DNA (sonicated and denatured, Sigma Chemical Company, St. Louis, MO) was added to the slides for 1 hour at 42°C. Excess prehybridization fluid then was removed by blotting of the edges of the liquid with a clean tissue or paper towel, and 7 ng/μl probe (diluted in prehybridization solution) was added and the slides were kept at 42°C overnight. Negative control included slides with 10 ng/μl sense probe. As positive controls, paraffin sections from rabbit aorta were used.

The slides then were rinsed in 2 × SSC for 10 minutes at room temperature, followed by a rinse in 1 × SSC for 10 minutes at 37°C. The ABC method was employed as described above. Slides were counterstained with methyl green.

Results

Expression of TM in Normal Lung Tissue

Thrombomodulin was expressed on normal endothelial and mesothelial cells as well as a subpopulation of alveolar macrophages. Other types of cells in the lungs, including bronchial epithelium, mucous glandular epithelium, and pneumocytes, stained negatively with antibodies to TM. Reactive mesothelial cells isolated from pleural effusion also stained positively. The expression of TM in

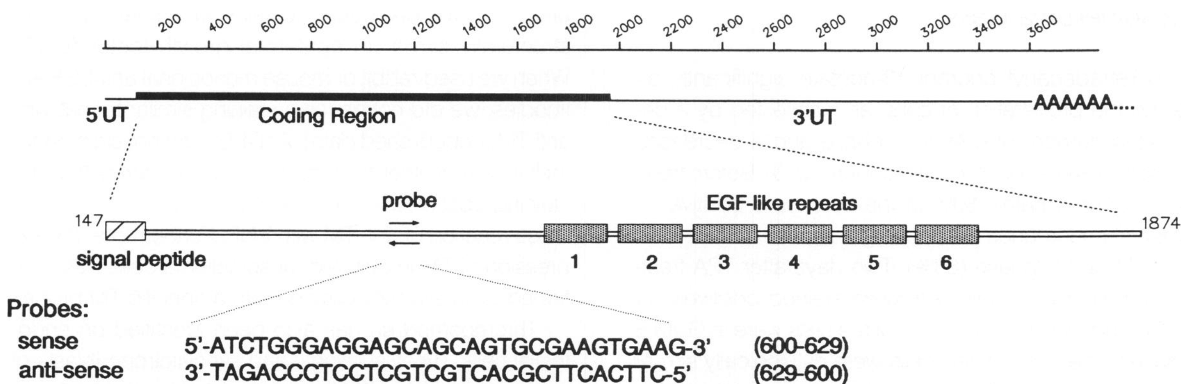

Figure 1. *TM antisense and sense probes were prepared based on the sequence 600-629. The sequence does not overlap with the EGF-like repeats.*

endothelial cells and mesothelial cells appeared to be localized on the cell surface.

Expression of TM, CEA, and Leu M1 in Mesotheliomas

All malignant pleural mesotheliomas stained positively with antibodies to TM (Figure 2). In addition to a surface distribution, abundant TM was also observed within the cytoplasm of mesothelioma cells. In 28 of the mesotheliomas, the staining with antibodies to TM was strongly positive. Three showed focally positive staining. The focal staining is thought to be due to poor fixation and subsequent loss of antigens. All of the specimens stained negatively with antibodies to CEA, and all but one (a biphasic mesothelioma) were stained negatively with antibodies to Leu M1. The biphasic mesothelioma had a weak, focal reactivity with antibodies to Leu M1.

The cells of the two cultured human mesothelioma cell lines (MS-1 and MS-2) were stained positively with antibodies to TM. Various percentages (10% to 20%) of cultured cells expressed TM on the surface as well as in the cytoplasm (Figure 2).

Expression of TM, CEA, and Leu M1 in Adenocarcinomas

Only four of the 48 adenocarcinomas stained positively with antibodies to TM (Figure 2). Three of the four showed minimal focal staining and one had intense positive staining. In all of these specimens, the endothelial cells, which served as an internal control, stained positively with antibodies to Leu M1. Most of the adenocarcinoma cells were stained positively with anti-CEA (79%) and anti-Leu M1 (60%).

Effects of TPA on Cultured Mesothelioma Cells

12-0-Tetradecanyl phorbol 13-acetate significantly affected the proliferation of cells, as manifested by a decreased number of cells in S phase and a decreased number of Ki-67–positive cells (Figures 2, 3). Before treatment, approximately 80% of the cells were positive for Ki-67, in accordance with the large number of MS-1 cells in G_2/M and S phase (66%). Two days after TPA treatment, only 30% of the cells were stained positively for Ki-67, and only less than 13% of the cells were in G_2/M/S phases. The TPA-treated cells were cytologically similar to reactive mesothelial cells, with a decreased nuclear/cytoplasmic ratio and slightly eccentric nuclei.

The expression of TM was markedly increased in approximately 60% of the TPA-treated MS-1 cells. Staining was located mainly on the surface of these cells, but a large number of cells also exhibited cytoplasmic staining. Double staining indicated that TM can be expressed both in Ki-67–positive and in Ki-67–negative cells, as well as in cells undergoing mitosis.

In Situ Hybridization for Detection of TM mRNA

Most cultured MS-1 cells exhibited weak nuclear staining when labeled with anti-sense probe (Figure 2). Dense paranuclear or cytoplasmic staining was observed in 15% of MS-1 cells, however; the number of TM-mRNA positive cells corresponded to that of cells stained by anti-TM. No staining was observed when sense probe was used.

Discussion

The differentiation of malignant pleural mesothelioma from pulmonary adenocarcinoma has been approached by many different methods, such as immunostaining with antibodies to vimentin, Leu M1, and B72.3. None of the single antibodies offers complete sensitivity and specificity, however. In this study, we noted that staining with anti-TM could be added to the battery, and that this could increase the sensitivity and specificity for the diagnosis of mesotheliomas. In our series, all mesotheliomas stained positively with anti-TM, yielding a sensitivity of 100%. In contrast, only four of 48 adenocarcinomas stained positively with anti-TM, yielding a specificity of 92%.

Thrombomodulin was first described by Esmon et al in 1981.[12] The anticoagulant activity of TM results from the activation of protein C and the subsequent action on factors Va and VIIIa, and from the binding of thrombin.[13,14] Thrombomodulin contains six domains that are structurally similar to epidermal growth factor (EGF). When we used rabbit or mouse monoclonal anti-EGF antibodies, we did not observe staining similar to that with anti-TM (unpublished data). Anti-EGF did not stain endothelial or mesothelial cells. It is thus apparent that the staining observed in mesothelioma cells is not due to a cross-reaction of anti-TM with EGF. Furthermore, the expression of TM in cultured mesothelioma cells was confirmed by *in situ* hybridization with a specific TM probe.

Thrombomodulin has also been identified on endothelial cells, synovial linings, and syncytiotrophoblasts of the placenta.[10] Anti-TM immunostaining has been shown to be an excellent test for vascular tumors, particularly

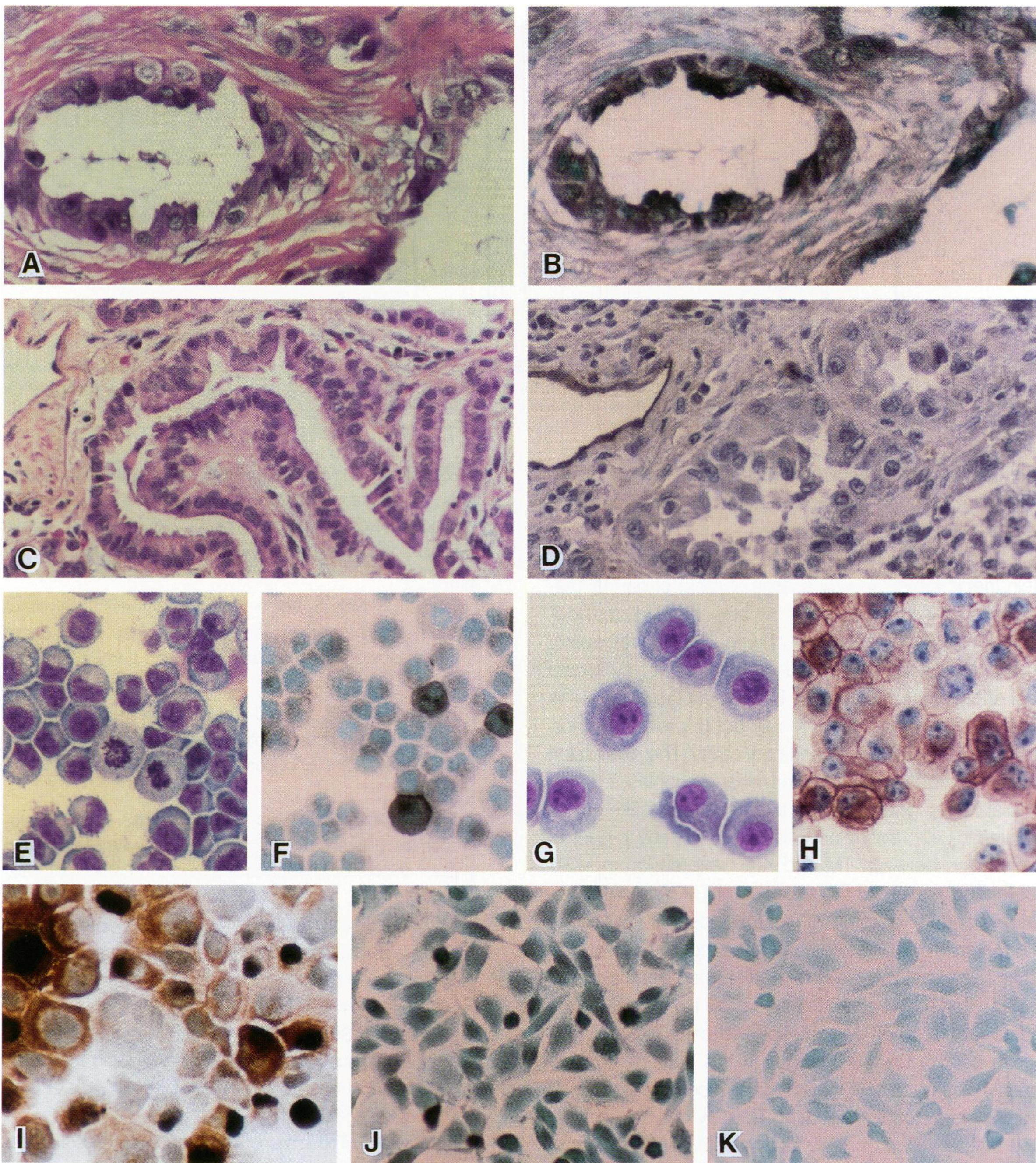

Figure 2. *Anti-TM showed positive staining in malignant pleural mesothelioma* (**A,B**), *but not in pulmonary adenocarcinoma* (**C,D**). *The staining in vascular endothelium served as an internal control* (**D**). *In MS-1 cells* (**E**), *anti-TM stained approximately 20% of the cells* (**F**). *Treatment of MS-1 cells with phorbol ester resulted in the differentiation of these cells* (**G**) *and in increased staining with anti-TM* (**H**). *Double staining of MS-1 cells with both anti-Ki-67 (detected with DAB-Ni, yielding gray-black positivity) and anti-TM (detected with AEC, yielding red-brown positivity) showed no relationship between cell cycle and TM expression (I). The expression of TM in MS-1 cells was confirmed by positive* in situ *hybridization with TM antisense probe* (**J**), *but not with the control TM sense probe* (**K**).

angiosarcomas,[15] and for choriocarcinomas.[16] The presence of TM in these two types of tumors does not interfere in any way with the usefulness of this antibody in aiding the diagnosis of mesotheliomas, because these tumors differ in their histopathology.

In this study, we also evaluated the expression of CEA and Leu M1 in adenocarcinoma. Seventy-nine percent of the adenocarcinomas stained positively with anti-CEA, and 55% stained positively with anti-Leu M1. None of the mesotheliomas stained with anti-CEA, but one showed minimal staining with anti-Leu M1. This finding suggests that CEA is more sensitive and specific than is Leu M1 for

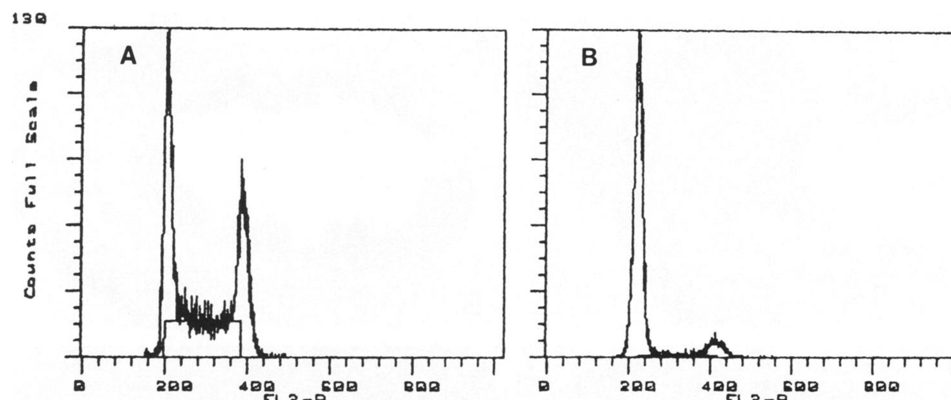

Figure 3. *Cell-cycle analysis of MS-1 cells stained with propidium iodide* (**A**). *Treatment with phorbol ester caused a marked decrease in the number of cells in S and G_2/M phase* (**B**). *The percentage of cells in G_0/G_1 phase in control culture was 35%, and that in TPA-treated culture was 66%. The decrease in number of cells in G_2/M/S phase coincides with the differentiation of MS-1 cells. The* x *axis represents the intensity of staining fluorescence, and the* y *axis, the number of cells stained (linear scale).*

the diagnosis of adenocarcinomas. Our results indicate that anti-CEA and anti-Leu M1 are not quite as sensitive as had previously been reported.[2]

Thrombomodulin appears to be a major surface protein that is expressed by a large number of normal mesothelial cells and mesothelioma cells in tissue. Immunoelectron microscopic study showed that TM was evenly distributed on the cell surface, including all microvilli (data not shown). The function of TM in mesothelial cells has not yet been determined; it may act to prevent clot formation in the pleural or peritoneal cavity. The expression of TM in large numbers of normal and neoplastic mesothelial cells suggests that the expression is not likely to be related to cell proliferation. This is confirmed by the lack of correlation between TM and Ki-67 expression in MS-1 cells. The reason for the absence of TM from a considerable number of the cultured MS-1 cells is not known. However, TM expression can be induced by TPA, which is known to activate several protein kinases in cells. The MS-1 cells may be a suitable model for studies of the gene regulation of TM in mesothelial cells.

In conclusion, our findings in this series of experiments clearly indicate that immunohistochemical staining with a monoclonal antibody to TM may be very useful in the differentiation of malignant mesothelioma from adenocarcinoma. The method is highly sensitive and specific, and it can be applied to routinely processed, formalin-fixed, and paraffin-embedded tissue sections. With this method, however, one cannot differentiate malignant mesothelioma from reactive mesothelial lesions.

References

1. Tron V, Wright JL, Churg A: Carcinoembryonic antigen and milk-fat globule protein staining of malignant mesothelioma and adenocarcinoma of the lung. Arch Pathol Lab Med 1987, 111:291–293
2. Wick M, Loy T, Mills S, Legier J, Manivel J: Malignant epithelioid pleural mesothelioma vs. peripheral pulmonary adenocarcinoma: A histochemical, ultrastructural, and immunohistologic study of 103 cases. Hum Pathol 1990, 21:759–766
3. Frierson H, Mills S, Legier J: Flow cytometric analysis of ploidy in immunohistochemically confirmed examples of malignant epithelial mesothelioma. Am J Clin Pathol 1988, 90:240–243
4. Donna A, Betta P, Jones J: Verification of the histologic diagnosis of malignant mesothelioma in relation to the binding of an antimesothelial cell antibody. Cancer 1989, 63:1331–1336
5. Cagle P, Truong L, Roggli V, Greenberg S: Immunohistochemical differentiation of sarcomatoid mesotheliomas from other spindle cell neoplasms. Am J Clin Pathol 1989, 92:566–571
6. Kawai T, Suzuki M, Torikata C, Suzuki Y: Expression of blood group-related antigens and Helix promatia agglutinin in malignant pleural mesothelioma and pulmonary adenocarcinoma. Hum Pathol 1991, 22:118–124
7. Warnock ML, Stoloff A, Thor A: Differentiation of adenocarcinoma of the lung from mesothelioma: Periodic acid-Schiff, monoclonal antibodies B72.3 and Leu M1. Am J Pathol 1988, 133:30–38
8. Ordonez NG: The immunohistochemical diagnosis of mesothelioma: Differentiation of mesothelioma and lung adenocarcinoma. Am J Surg Pathol 1989, 13:276–291
9. Wen D, Dittman W, Ye R, Deaven L, Marjerus P, Sadler J: Human thrombo-modulins: Complete cDNA sequence and chromosome localization of the gene. Biochemistry 1987, 26:4350–4357
10. Maruyama I, Bell C, Majerus P: Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncitiotrophoblasts of human placenta. J Cell Biol 1985, 101:363–371
11. Hsu SM, Hsu PL, Zhao X, Kao-Shan CS, Whang-Peng J: Establishment of human mesothelioma cell lines (MS-1, -2) and production of a monoclonal antibody (anti-MS) with

diagnostic and therapeutic potential. Cancer Res 1988, 48: 5228–5236
12. Esmon NL, Owen WG, Esmon CT: Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J Biol Chem 1982, 25:859–864
13. Dittman W, Majerus P: Structure and function of thrombomodulin: A natural anticoagulant of blood. Blood 1990, 75: 329–336
14. Suzuki K, Kusumoto H, Deyashiki Y, Nishioka J, Maruyama I, Zushi M, Kawahara S, Honda G, Yamamoto S, Horiguchi S: Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. EMBO J 1987, 6:1891–1897
15. Yonezawa S, Maruyama I, Sakae K, Igata A, Majerus P, Sato E: Thrombomodulin as a marker for vascular tumors. Am J Clin Pathol 1987, 88:405–411
16. Yonezawa S, Maruyama I, Tanaka S, Nakamura T, Sato E: Immunohistochemical localization of thrombomodulin in chorionic diseases of the uterus and choriocarcinoma of the stomach. Cancer 1988, 62:569–576