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原　　著

Generation of Monoclonal Antibodies that Distinguish between Mesotheliomas and Other Tumor of the Lung

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Abstract

The accurate diagnosis of mesothelioma remains difficult despite advances of diagnostic technique. And specific monoclonal antibody (McAb) against mesothelioma have not been reported.

In an attempt to develop mesothelioma specific McAb(s), spleen cells from a mouse immunized with isolated tumor cells were fused to a drug resistant mouse myeloma cell lines. Over 200 hybridomas were assayed for their preferential reactivity with mesothelioma cell lines or mesothelioma tumor biopsy tissues. Two monoclonal antibodies 2A3 and 4E1 were identified that bound 6/7 of the mesotheliomas, tested, but did not bind to the majority, 11/13 (for 2A3) and 12/13 (for 4E1), of other lung tumor types. Based upon western blot analysis of one and two-dimensional gels and upon the distribution pattern of the antibody recognized molecule in mesotheliomas and non-mesothelioma lung tumors, 2A3 binds to the cell adhesion molecule CD44. While the specificity of 4E1 has not yet been unequivocally established it appears to recognize a variant form of the CD44 molecule.

Introduction

Malignant mesothelioma (MM) commonly occurs in person who have a history of asbestos exposure. The incidence of MM in the U.S. and also in Japan is curently increasing in men. MM is also beginning to occur more frequently in women and in those who have no history of exposure to asbestos[1)].

The accurate diagnosis of MM remains difficult despite advances in diagnostic technique. Several McAbs have been reported for this purpose such as anti-Vimentin, anti-Leu-M1, anti-carcinoembryonic antigen, 44-3A6, B72.3 and Ber-EP4[2-9)]. These McAbs will be helpful in pathologic diagnosis, but none of them are specific to MM. In an attempt to develop MM specific McAb in our laboratory, two McAbs, 2A3 and 4E1, were produced and screened for specificity and the antigens recognized by these antibodies were charactrized.

Key words: Monoclonal Antibody, Mesothelioma, CD44
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Material and Methods

Cell Lines and Tissues

Established mesothelioma cell lines, MS-1 and MS-2, were obtained from *Dr. Su-Ming Hsu*, University of Texas Health Science Center. All the pulmonary carcinoma series cell lines were obtained from Tokyo Medical College, Tokyo, Japan. QU-DB was obtained from *Dr. Susan P. Coles,* Dept. of Microbiology and Immunology, Queens University, Kingston, Ontario, Canada. Normal mesothelial cells (AX-32) were obtained from *Dr. Cannistra,* The Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA. The 1E12, 2E9 and LA10 cell lines were established in our laboratory.

Fresh surgical specimens of MM were obtained from The Division of Thoracic Surgery and Oncology, Roswell Park Cancer Institute, Buffalo, NY. Tumor cells were collected by mechanical cell separation methods, washed twice with Delbecco's Modified Essential Medium (DMEM) and then resuspended in DMEM as tumor biopsy tissue.

Immunized tumor was prepared as above from metastatic MM of the skin. Cells were also frozen in RPMI 1640 containing 10% fetal bovine serum, 50 mM 2-ME, 2 mM L-glutamine, 20 unit/ml penicillin, 20 mg/ml streptomycin (Complete Medium) with 20% DMSO for later immunization.

Tissue Culture Cells

From tumor tissue:

Minced tumor tissues were placed in small petri dish with Complete Medium. After 10 to 14 days, attached cells were harvested by trypsin treatment (0.05% trypsin, 0.04% EDTA in normal saline) and expanded for further process.

From pleural effusion:

200 ml of pleural effusion was centrifuged and collected cells were placed into 12 well plate. Approximately 1 week later attached cells were harvested by trypsin treatment and expanded for further process.

Production of Monoclonal Antibodies

Two antibodies, 2A3 and 4E1 were produced using hybridoma techniques as described previously[10]. BALB/C mice were immunized by intraperitonal injection of 5×10^6 tumor cell suspension. The mice were boosted one month later by the same immunization protocal. 48 hours later, the mice were sacrificed and spleen cells were fused to the myeloma cell line P3x63Ag8.653.

McAbs 2A3 and 4E1 were selected from over 200 other hybridomas in preliminary screening and used for further screening.

Immunocytoadherence Assay

Culture cells were harvested by trypsin treatment and washed twice with phosphate buffer saline (PBS). Cells were resuspended in PBS at an adequate number for each cell lines and incubated with McAb for 20～60 minits at 4°C. Then washed three times with cold PBS and added rabbit anti-mouse Ig conjugated sheep red blood cells. Samples were examined by light microscopy and percentage of rosette forming cell was calculated[11]. Established MM cell line, fresh tumor cell suspension and primary tissue culture cell of MM and also established non-mesothelioma tumor cell lines were used in this assay.

Western Blot of an One Dimensional SDS-PAGE

MS-1 cell pellet (2×10^8 cells) was homogenized in 2 ml of hypotonic lysis buffer (50 mM manni-

tol, 5 mM Hepes, 0.5 unit/ml Aprotinin, pH 7.4) by forcing it 3 times through 18-G needle and twice through 25-G needle. 10 mM $CaCl_2$ was then added and passed through 25-G needle once more. Mixture was centrifuged for 12 minits at 1450xg to eliminate the nuclear, mitochondrial and microsomal fraction. The resulting supernatant, containing the plasma membrane vesicles, was centrifuged for 30 minits at 90,000xg. The white translucent plasma membrane pellet was resuspended in PBS. This solution was mixed in 2x unreduced Laemmli sample buffer[12], boiled for 2 min. and electrophoresed in preparative 10% polyacrylamide minigels (Bio Rad, Rhichmond, CA). Proteins were blooted to polyvinylidenedifluoride membrane (Immobilon-Millipore, Bedford, MA) and placed in blocking buffer (Tris buffer saline, 10% {w/v} nonfat dry milk, 0.5% Tween-20, 0.5% normal goat serum) for over 3 hours. Test strips were cut from the blot and incubated for 3 hours at room temperature with tissue culture supernatant of 2A3, 4E1, anti CD44 Ab and anti VLA-β_1 Ab diluted in blocking buffer to 2 times, and ascites harvested from hybridoma (2A3, 4E1) injected BALB/c mice were also diluted to 2 μg/ml and incubated. After washing in blocking buffer, test strips were incubated with peroxidase conjugates of goat anti-mouse IgG+A+M (Zymed) diluted 1:1000 in blocking buffer for 1 hour at room temperature. After incubation, test strips were washed 3 times in TBS with 0.5% Tween-20 before development using enhanced chemiluminescence (ECL, Amersham).

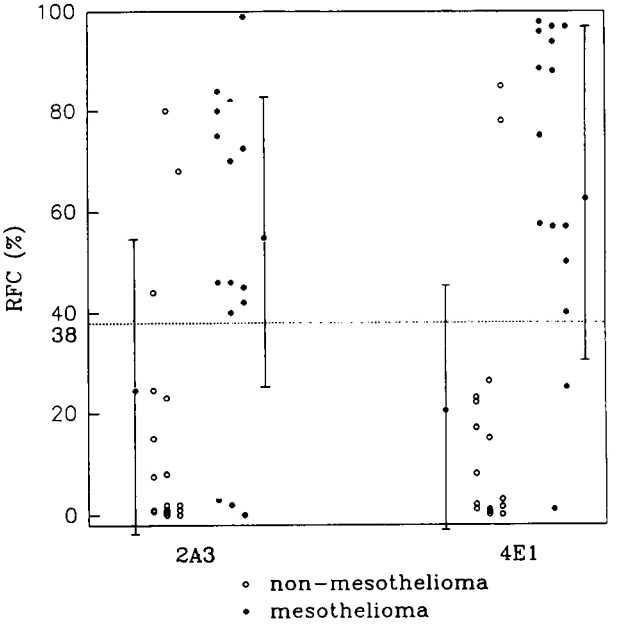

Fig. 1 Specificity of Monoclonal Antibodies 2A3 and 4E1
The immunocytoadherence screening results was summarized in this figure and analyzed statistically. Each dot represents a different cell line.
2A3 and 4E1 bind preferentially to mesothelioma (2A3, p=0.0114 and 4E1, p=0.0146).

Western Blot of a Two-Dimensional SDS-PAGE

The plasma membrane fractions were dissolved in O'Farrell 2-dimensional lysis buffer[13]. The proteins were separated in the first dimension by isoelectric focusing (Mini-PROTEAN II 2-D Gell, Bio-Rad, Ritchmond, MA) and second dimension by 10% polyacrylamide mini gel. The gels were subsequently analyzed by western blot.

Results

Production of Monoclonal Antibodies

Over 200 hybridomas cultures were screened by immunocytoadherence assay using MS-1 and MS-2 as the target cell. From this preliminary screening, two clones, 2A3 and 4E1, that selectively bound strongly to mesothelioma cells were selected. The immunogloblin isotype of 2A3 and 4E1 were determined by SBA Clonotyping System III (Southern Biotechnology Associates, Inc. Birmingham, AI). 2A3 was isotyped as IgG3, kappa and 4E1 was determined to be IgG1, kappa.

Preferential Binding of Antibodies to Mesothelioma:

2A3 and 4E1 were reacted with malignant mesothelioma and non-mesothelioma tumors (Fig. 1). The reactivity of the two antibodies with mesotheliomas is significantly different from the reactivity with non-mesothelioma tumors (i.e a preferential reactivity with the mesothelioma 2A3, $p=0.0114$ and 4E1, $p=0.0146$). 2A3 and 4E1 bound to the majority of fresh mesothelioma biopsy tissue i.e. 6/7 (2A3) 6/7 (4E1) and to most of the mesothelioma tissue culture cells tested 4/6 (2A3)

Table 1 Binding of Monoclonal Antibodies to Malignant Mesothelioma

		2A3	4E1
Cell Line	*MS-1*	84.0	96.0
	MS-2	82.0	94.0
	Positive Ratio	2/2	2/2
Tumor Cell Suspension	*P-1*	72.5	57.5
	P-2	75.0	88.5
	P-3	46.0	57.0
	P-4	3.0	1.0
	P-5	40.0	97.0
	P-6	70.0	75.0
	P-7	45.0	40.0
	Positive Ratio	6/7	6/7
Primary Tissue Culture Cell	*P-3CT*	46.0	57.1
	P-8CT	80.0	88.0
	P-9CT	2.0	98.0
	P-9PE	99.0	97.0
	P-10CT	0.0	50.0
	P-11PE	42.0	25.0
	Positive Ratio	4/6	5/6
Total Positive Ratio		12/15	13/15

Tumor Cell Suspension and Primary Tissue Culture Cell were isolated from different patient; CT: isolated from tumor tissue, PE: isolated from pleural effusion
Number in each colum means the percentage of Rosettte Forming Cell (RFC) >38% RFC scored as positive binding

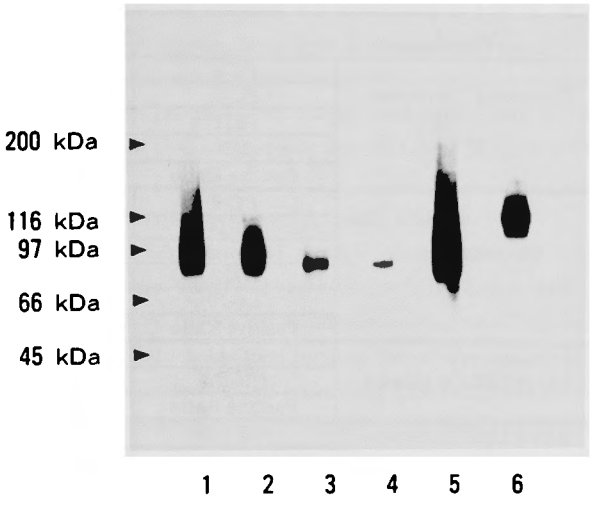

Fig. 2 Western Blot Analysis of One Dimensional SDS-PAGE
1: 2A3 Tissue Culture Supernatant (TCS), 2: 2A3 ascites, 3: 4E1 TCS, 4: 4E1 ascites, 5: 3C9 TCS, 6: 5D9 TCS (anti-β_1)
The plasma membrane fraction extracted from MS-1 cells were analyzed by western blot. Both McAb 2A3 and 4E1 bind the target band in unreduced condition. 2A3 is bound to a molecule with an estimated molecular weight of 80～94 kDa and 4E1 is boud to a molecule with an estimated molecular weight of 82～96 kDa. Anti-CD44 Ab binds molecules with an estimated molecular weight of to 78～160 kDa molecule.

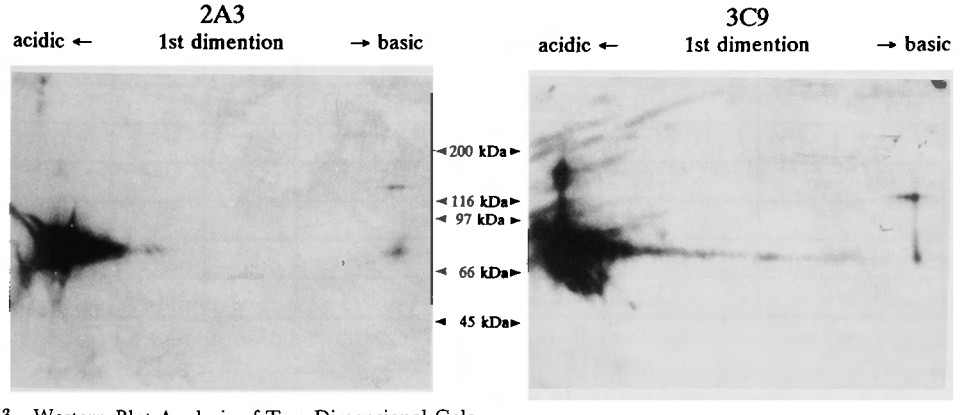

Fig. 3 Western Blot Analysis of Two-Dimensional Gels
The plasma membrane fraction extracted from MS-1 cells were analyzed by 2-dimensional gel erectrophoresis to compare McAb 2A3 and anti-CD44 Ab. 2A3 target molecule is CD44.

Table 2 Binding of Monoclonal Antibodies to Non-mesothelioma Tumor

Lung Carcinoma		2A3	4E1
Squamous Carcinoma	*PC-1*	7.5	2.0
	PC-3	0.7	1.0
	1E12	1.0	0.0
	2E9	1.0	0.0
	Positive Ratio	0/4	0/4
Adenocarcinoma	*PC-9*	24.5	23.0
	PC-14	44.0	0.5
	Calu-3	1.0	26.3
	A427	15.0	85
	LA-10	2.0	3.0
	Positive Ratio	1/5	1/5
Large Cell Carcinoma	*PC-13*	0.0	17.0
	QU-DB	0.0	1.0
	Positive Ratio	0/2	0/2
Small Cell Carcinoma	*NCI-H69*	0.5	1.5
	Positive Ratio	0/1	0/1
Alveolar Carcinoma	*A549*	80.0	8.0
	Positive Ratio	1/1	0/1
Other Carcinoma			
Gastric Carcinoma	*KATO-III*	2.0	15.0
Epidermoid Carcinoma	*A431*	23.0	78.0
Neurosarcoma	*SK-N-SH*	68.0	22.0
Fibrosarcoma	*HT1080*	8.0	0.0
	Positive Ratio	1/4	1/4
Total Positive Ratio		3/17	2/17

>38% RFC scored as positive binding

Table 3 Distribution Pattern of Molecule Recognized by 2A3 and 4E1 Resembles that of Cell Adhesion Molecule CD44

Cell Line	Histology	Monoclonal Antibodies		
		2A3	*4E1*	*3C9*[1]
MS-1	Malignant Mesothelioma	+[2]	+	+
MS-2	Malignant Mesothelioma	+	+	+
PC-14	Lung Adenocarcinoma	+		+
AX 32	Normal Mesothelial Cell	+	+	+
IMR-90	Fetal Lung Fibroblast	+	+	+
WI-38	Fetal Lung Fibroblast	+	-	+
1E12	Lung Squamous carcinoma	-[3]		
LA10	Lung Adenocarcinoma			-

1. 3C9 : A monoclonal antibody specific for human CD44 (G. Dougherty)
2. + : > 38% RFC
3. : < 38% RFC

and 5/6 (4E1) (Table. 1).

These antibodies did not bind to the majolity 11/13 (2A3) and 12/13 (4E1) of non-mesothelioma lung tumor types i.e. squamous cell, adeno, large cell, and small cell carcinoma. Other carcinomas including a gastric carcinoma and fibrosarcoma were determined to be negative while a neurosarcoma expressed the ligand recognized by 2A3 and epidermoid carcinoma did so by 4E1 (Table. 2).

According to the mean value of two groups, we decided over 38% of RFC-average of the mean of these groups-as positive.

Characterization of Molecules Recognized by 2A3 and 4E1:

The reactivity pattern of both 2A3 and 4E1 were similar to the anti-CD44 adhesion molecule specific antibody (3C9). That is the positive reactivity to MM and normal mesothelial cell, fetal lung fibroblast, and also the negative reactivity to adonocarcinoma of the lung (Table. 3).

2A3 and 4E1 recognized the SDS-denatured form of the native molecule they recognized in the RFC assay. 2A3 recognized a molecule with an estimated molecular weight of 80～94 kDa and 4E1 bound to a molecule with an estimated molecular weight of 82～96 kDa in the western blots of the unreduced gels (Fig. 2). Western blot analysis of a two dimensional SDS-PAGE of the detergent solubilized membrane fraction of MS-1 cells revealed that 2A3 and the anti-CD44 antibody 3C9 recognized molecules of the same molecular weight and isoelectric point (Fig. 3).

Discussion

Since the dismal median survival of MM is approximately 11 months, so to improve the prognosis of MM, it is very important to make a precise diagnosis of MM and to start treatment as soon as possible. Several McAbs (anti-vimentin, anti-Leu-M1, anti-carcinoembryonic antigen, B72.3, and Ber-EP7 etc) were reported for diagnosis of MM, but these McAbs are used mainly in immunohistostaining of surgical specimen and most of them are not specific to MM. Pleural biopsy may not produce enough tissue to enable a firm diagnosis. We try to establish more predictal diagnotic method such as blood test before surgical treatment.

For this purpose, we attempt to develop malignant mesothelioma specific McAb, two McAbs were developed and screened for their specificity and selectivity. McAbs 2A3 and 4E1 recognize the molecule that was expressed more frequently on malignant mesothelioma cells than on non-mesothelioma tumor cells.

Established MM cell line, fresh tumor cell suspension and primary tissue culture cell mostly expressed both 2A3 and 4E1. Just two of those (P-9CT and P-10CT) expressed only 4E1. Even from same patient, primary tissue culture cells from tumor and pleural effusion (P-9CT, P-9PE) showed different reactivity with 2A3 and 4E1. It may be because of heterogeneity of MM.

McAb 2A3 did not bind exclusively to most of non-mesothelioma tumor cell lines except three cell lines (PC-14, A549 and SK-N-SH). 4E1 did not bind to those cell lines except A427 and A431.

In the course of these studies, we determined that the expression of the molecule recognized by 2A3 and 4E1 on different mesotheliomas and non-mesothelioma lung tumors was very similar, and after screening several different antibodies of known specificities including anti-CD44 McAb. Because a high level of hyaluronic acid in the pleural effusion is found more commonly in MM than in other tumors, MM was thought to have some expression of CD44. Both 2A3 and 4E1 showed a similar pattern of the reactivity to anti CD-44 McAb (3C9). The possibility that 2A3 and 4E1 were recognizing determinants on the cell adhesion molecule CD44 was further explored by western blot

analysis.

Both McAbs 2A3 and 4E1 were shown to bind a band in a western blot of an one dimensional SDS-PAGE of the cell lysate of MS-1 cells. These same antibodies both failed to recognize the antigenic determinant when the cell lysates were reduced with 2 ME prior to SDS-PAGE.

Both the molecular weight and the sensitivity to 2 ME (due to intra-chain disulfide bonds) are consistent with the motion that thse antibodies were recognizing a standard or variant form of the CD44 molecule.

Target molecule of 2A3 was not expressed exclusively on malignant mesothelioma cells since it was also expressed on normal mesothelial cells and fetal lung fibroblast cells. This result is consistent since the target molecule of 2A3 is a CD44 molecule that is expressed on many cell types such as leukocyte, erythrocyte, fibroblast, myeloid cell, smooth muscle, etc[14]. CD44 is an integral membrane glycoprotein, originally described as a homing receptor of lymphocytes. Beside the involvement of CD44 in adhesion of lymphocytes to specialized endothelial cells, CD44 is known to bind to the extracellular matrix components hyaluronic acid, fibronectin, and collagen, suggesting roles in the interaction between cells and extracellular matrix[15]. CD44 has several variant forms as well as the standard form. The different CD44 proteins have the same sequences at their ends but differ in the middle, which is located just outside the cell membrane. CD44 variant is an essential molecule in the activation of immature lymphocytes into mature immune cells in the lymph nodes. Several variants of CD44 with additional extracellular domains have been detected in a variety of tissues and frequently on tumor cells[16].

One of the variant forms has been reported to be causally associated with metastasis[14–20]. The target molecule of 4E1 has not been established but the possibility that this antibody recognized a variant form of CD44 was suggested. This means that mesothelioma cells have very adhesive charactor between cells, also cell and extracellular matrix and it may cause extensive tumor growth since it do not metastasize to another organ.

We anticipate that the preferential reactivity of 2A3 and 4E1 will be useful in the discrimination between mesotheliomas and other tumors of the lung, and the preferential expression of the 2A3 and 4E1 target ligands on human mesotheliomas may reveal insights regarding the growth patterns and metastatic potential of thse tumors.

We conclude that 2A3 recognizes the standard form of CD44 and while we cannot unequivocally establish the identity of the molecule recognized by 4E1, we postulate that this may present a variant form (either splice variant or different glycosylation pattern) of CD44. And 2A3 is thought to be useful in early detection of MM.

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和文抄録

悪性中皮腫と他の肺悪性腫瘍との鑑別のためのモノクローナル抗体の作成

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悪性中皮腫の診断は困難なことが多く，特に，肺の腺癌との鑑別は困難である．また悪性中皮腫に特異的なモノクローナル抗体もいまだ報告されていない．

そこで，悪性中皮腫に特異的モノクローナル抗体の作成を試みた．

悪性中皮腫の新鮮な手術標本から得られた腫瘍細胞で BALB/C マウスを免疫し，脾細胞を採取した．同脾細胞をマウスミエローマ細胞と融合し，ハイブリドーマを作成した．200以上のハイブリドーマより，2つのモノクローナル抗体 2A3 と 4E1 を1次スクリーニングによって選択し，Immunocytoadherence Assay を行った．2A3, 4E1 ともに，悪性中皮腫の継代培養細胞に対して陽性で，新鮮腫瘍細胞浮遊液に対しては7例中6例が陽性であった．初代培養細胞に対しては 2A3 が6例中4例，4E1 が6例中5例に陽性を示した．悪性中皮腫以外の腫瘍細胞に対しては大多数が陰性で，2A3 は13例中2例，4E1 は13例中1例のみが陽性であった．

2A3 と 4E1 の標的分子の解析のために Western blot を行ったところ，これら2つの抗体が CD44 の standard または variant form を認識している可能性が示唆された．さらに，2次元の Western blot で 2A3 は抗 CD44 抗体と同じ分子量，同じ等電点を持つ分子を認識していることが判明した．