A Novel Monoclonal Antibody-defined Antigen Which Distinguishes Human Non-Small Cell from Small Cell Lung Carcinomas

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ABSTRACT

Spleen cells from BALB/c mice hyperimmunized with the human epidermoid lung carcinoma cell line T222 were fused with NS-1 mouse myeloma cells to produce monoclonal antibodies to human lung cancer antigens. Hybridoma culture supernatants were tested by an enzyme-linked immunosorbent assay for reactivity against a panel of human lung tumor cell lines. Supernatant from hybridoma EA1 (immunoglobulin G1) displayed strong reactivity with four of four non-small cell lung carcinomas but did not react with three of three small cell lung carcinoma (SCLC) cell lines. This hybridoma was cloned by limiting dilution and utilized to generate ascites antibody for subsequent immunohistochemical and antigen characterization studies. Evaluation of fresh frozen tumor tissue sections by immunoperoxidase staining methods revealed EA1 reactivity with the vast majority of non-SCLC tumor tissues. EA1 also stained bronchial epithelium and other benign and malignant epithelial tissues. The EA1 antigen was determined to have a molecular weight of 75,000 by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human non-SCLC tumor extracts. These data imply that EA1 recognizes a novel antigen expressed by non-SCLCs and other epithelial tissues. The absence of EA1 reactivity with SCLCs suggests that this monoclonal antibody may find future application in distinguishing non-SCLC from SCLC and prove useful in furthering our understanding of the histogenesis of lung carcinomas.

INTRODUCTION

Carcinomas of the lung represent one of the leading causes of cancer mortality in industrialized nations (1). Proper therapeutic management of patients with this disorder is dependent upon the accurate assessment of tumor histology (1, 2). Non-small cell lung carcinomas are relatively resistant to chemotherapy and radiation, but they are curable by surgical resection in a small subset of patients without demonstrable metastatic disease (3). Conversely, SCLCs (4) are very sensitive to chemotherapy and radiation, but are not amenable to cure by surgical resection as these tumors have invariably metastasized to distant sites at the time of initial diagnosis (3, 4). These major histological types of lung cancer can usually be distinguished on the basis of light microscopic evaluation of tumor biopsy specimens (2). However, differential diagnosis by morphological criteria is not always satisfactory, and other diagnostic techniques including electron microscopy and special stains for keratin, mucin, and neuroendocrine hormones may be useful in confirming the diagnosis of selected cases (1, 2, 5). Recently, several groups have developed MoAbs reactive with distinct subsets of human lung carcinomas which may advance our ability to diagnose and classify lung cancers (6–18). We describe in this paper a MoAb to a novel epithelial antigen expressed by non-SCLCs and not detected in SCLCs.

MATERIALS AND METHODS

Monoclonal Antibody Production. To generate MoAbs to lung cancer-associated antigens, BALB/c mice were hyperimmunized i.p. with 1 × 10⁷ live cells of the human epidermoid lung carcinoma cell line T222 at biweekly intervals for 4 wk. A final booster immunization was given 2 wk thereafter, and 72 h later the animal was sacrificed, and the spleenocytes were fused with NS-1 myeloma cells using the method of Galfre et al. (19). Undiluted hybridoma culture supernatants were tested for reactivity against a panel of human non-SCLC and SCLC cell lines utilizing an ELISA developed in our laboratory (20). Briefly, 2 × 10⁶ cells of the test carcinoma cell lines were placed in filter paper discs in a specially designed 96-well incubator chamber/filtration manifold device (V and P Enterprises, La Jolla, CA) and then incubated for 30 min with the hybridoma supernatants. The plates were washed, incubated with goat anti-mouse IgG antisera conjugated to horseradish peroxidase (Tago, Burlingame, CA), and washed again, and the enzyme reaction was developed by the addition of H₂O₂ and O-phenylenediamine. Positive wells turn brown in color, and the degree of antibody binding was quantified by measuring the absorbance of each well on an automated MR5-80 Microelisa reader (Dynatech, Santa Monica, CA). Cells incubated with NS-1 supernatant containing irrelevant murine immunoglobulin served as a negative control. Supernatants from one hybridoma, termed EA1, reacted with non-SCLC cell lines but not with SCLC cell lines and was chosen for further study. This hybridoma was cloned by limiting dilution and utilized to generate ascites antibody for subsequent immunohistochemistry and antigen characterization studies. EA1 was shown to be an IgG1 by commercially available murine immunoglobulin isotype radial immunodiffusion plates (Meylo Laboratories, Springfield, VA). EA1 was then tested for reactivity against a large panel of human cell lines by ELISA. NS-1 supernatant containing an irrelevant murine IgG1 myeloma protein (MOPC-21; Litton Bionetics, Kensington, MD) was used as a negative control. Tests were performed in duplicate, and the difference between the averaged absorbance at 490 nm for test and control wells was taken as the measure of the degree of specific EA1 binding to the human cell lines.

Immunohistology. Fresh frozen tissue specimens were evaluated for EA1 expression utilizing standard immunoperoxidase staining methods (21). Briefly, air-dried acetone-fixed cryostat sections were incubated with a 1/500 dilution of EA1 ascites for 30 min at room temperature and then washed extensively with phosphate-buffered saline. The sections were then incubated for 30 min with horseradish peroxidase-conjugated goat anti-mouse IgG antiserum (Tago, Inc., Burlingame, CA), washed with phosphate-buffered saline, and followed by the addition of diaminobenzidine chromagen–H₂O₂ substrate to develop the peroxidase reaction. The sections were counterstained with hematoxylin, coverslipped, and evaluated for staining by light microscopy. An identically prepared irrelevant murine IgG MoAb ascites, handled as described above for EA1, served as a negative control and did not stain any of the tissue sections. Whenever possible, surgical tissue specimens were utilized in preference to autopsy specimens for immunohistochem-
ical studies. All non-SCLC tumors and 5 of 9 SCLCs were obtained at surgery. Of the remaining normal and malignant tissue specimens evaluated, only normal bladder, brain, and spinal cord were procured at autopsy. All autopsy tissues were less than 18 h old.

**Immunofluorescence Staining.** EA1 reactivity with the human lung epidermoid cell line Calu-1 was evaluated by immunofluorescence cytofluorometric methods (22) to further examine the cellular location of EA1 antigen expression. Briefly, a single cell suspension of Calu-1 cells was incubated with a 1/500 dilution of EA1 or control irrelevant murine IgG ascites, washed, and then stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antisera. These specimens were analyzed for cell surface membrane EA1 reactivity utilizing an Ortho Cytofluorograph 50. Slides of the stained Calu-1 cells were also examined with an immunofluorescence microscope (Carl Zeiss, New York, NY).

**Antigen Characterization.** The molecular weight of the EA1 antigen was determined by immunoprecipitation and SDS-PAGE of human lung carcinoma cell line extracts. Briefly, 5 x 10⁶ non-SCLC (Calu-1) and control SCLC (T293H) cells were intrinsically labeled with 100 μCi of [³⁵S]methionine for 18 h. The cells were lysed for 1 h in a Nonidet P-40 detergent buffer (0.5% Nonidet P-40/1 mM polymethylsulfonylfluoride/5 mM EDTA/50 mM Tris, pH 7.4). The extracted macromolecules were ultracentrifuged (100,000 x g for 1 h), absorbed with an irrelevant murine ascites (Ehrlich's) overnight at 4°C to minimize subsequent nonspecific interactions, and washed twice by microcentrifugation. The supernatants were incubated with EA1 or an irrelevant control murine IgG ascites (PSB535) for 2 h at 4°C and precipitated with Pansorbin (Calbiochem, San Diego, CA). The samples were then prepared and subjected to SDS-PAGE as described (23). The molecular weight of the EA1 antigen was estimated by comparing gel autoradiographs and scintillation profiles of 1-mm gel slices against known molecular weight standards.

**RESULTS**

The results of EA1 reactivity with human tumor cell lines by ELISA are depicted in Fig. 1. EA1 displayed significant binding to 4 of 4 lung epidermoid and lung adenocarcinoma cell lines and was essentially unreactive with 4 of 4 cell lines established from lung tumors with SCLC characteristics. EA1 reacted to a lesser extent with other carcinomas of epithelial origin but was unreactive with skin fibroblast, melanoma, and lymphoid cell lines.

**CELL LINE**

<table>
<thead>
<tr>
<th>T293H</th>
<th>SK-MES-1</th>
<th>T222</th>
<th>CALU-1</th>
<th>M-103</th>
<th>CALU-4</th>
<th>T293H</th>
<th>NCI-69</th>
<th>A431</th>
<th>T84</th>
<th>350 Q</th>
<th>HELA</th>
<th>LNCAP</th>
<th>M21</th>
<th>WIL-2</th>
<th>B392</th>
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Expression of the EA1 antigen by malignant and normal tissues was evaluated by immunoperoxidase staining of cryostat tissue sections. The results of EA1 reactivity with malignant tissues are listed in Table 1. EA1 stained nearly all non-SCLCs evaluated (44 of 45). In most instances EA1 strongly stained the majority of tumor cells. Heterogenous EA1 reactivity with less than 50% EA1⁺ malignant cells was observed in a minority of cases. Three cases of non-SCLC displayed weak EA1 staining but were considered positive relative to their respective negative controls. EA1 did not stain any of the SCLCs tested (0 of 9). Representative EA1 reactivity with non-SCLC and SCLC tumors is shown in Fig. 2. EA1 also reacted strongly with breast and prostatic adenocarcinomas but displayed equivocal or no reactivity with colon carcinoma tissues. The reactivity of EA1 with normal tissues (Table 2) paralleled the staining of tumor specimens with respect to both tissue distribution and staining intensity. EA1 staining was detected with lung (bronchial and alveolar), breast, and prostatic epithelium, while minimal or no EA1 reactivity was observed with normal colon or gastric mucosal elements. EA1 also stained hepatic bile ducts, pancreatic exocrine cells, renal tubules, bladder epithelium, and skin epidermis. EA1 did not react with pancreatic islet cells, renal glomeruli, nor with thyroid, neural, or hematopoietic tissues.

The character of EA1 immunohistochemical staining in malignant and normal tissues was consistent with cytoplasmic expression of the EA1 antigen (Fig. 2). To evaluate EA1 cell surface membrane reactivity, a cell suspension of the lung epidermoid cell line Calu-1 was examined by immunofluorescence microscopy and cytofluorometry. Cytofluorometric and microscopic analyses revealed cell surface expression of the EA1 antigen (Fig. 3).

The molecular weight of the EA1 antigen was determined by immunoprecipitation and SDS-PAGE. EA1 precipitated a Mr 75,000 antigen from the human lung epidermoid cell line Calu-1 but not from the SCLC cell line T293H (Fig. 4).

**DISCUSSION**

Classification of human lung carcinomas based upon tumor histology has proven useful in the clinical management of patients, in furthering our understanding of the biology of lung tumors, and in guiding the development and evaluation of novel therapeutic protocols (1–5). However, the histological classification of individual cases of lung carcinomas may often be problematic (5). We have developed a MoAb termed EA1 which may complement morphological criteria in the classification of human lung carcinomas. EA1 identified a novel Mr 75,000 antigen expressed by the non-SCLCs evaluated in this investigation. The EA1 antigen was not detected by a variety of techniques on fresh tumor tissues or cell lines derived from...
SCLCs. These findings suggest that the EA1 antigen is expressed by non-SCLCs but not by SCLCs and that the EA1 MoAb may find application in distinguishing these clinically significant subtypes of lung cancer. Evaluation of a larger number of lung carcinoma specimens will be required to confirm the utility of EA1 for distinguishing non-small cell from small cell lung carcinomas.

The molecular weight and tissue distribution of the EA1 antigen indicate that EA1 is distinct from other MoAb-defined antigens expressed by non-SCLCs. Mulshine et al. (6) have produced MoAbs reactive with a Mr 31,000 antigen expressed by lung epidermoid and adenocarcinomas but absent from SCLCs. MoAbs reactive with lung epidermoid carcinomas but unreactive with lung adenocarcinomas and SCLCs have been reported by Brenner et al. (7) and Kyoizumi and coworkers (8). Varki et al. (9) and Radosevich et al. (10) have described MoAbs reactive with lung adenocarcinomas and unreactive with other types of lung cancers. Several investigators have developed MoAbs with apparent specificity for SCLC (11-13), and numerous MoAbs reactive with both non-SCLCs and SCLCs have been described (14-18).

The specificities of these MoAbs suggest that they will prove useful in distinguishing subtypes of lung carcinomas with known clinical significance and identify novel phenotypes for further investigation. Correlation of the reactivities of these MoAbs with lung tumors and normal tissues should also advance our understanding of the histogenesis and biology of pulmonary neoplasms. The expression of shared antigens (14-18) and the demonstration of ultrastructural and biochemical

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**Table 2.** EA1 immunohistological reactivity with benign tissues

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>No. of positive/no. tested</th>
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<tbody>
<tr>
<td>Lung</td>
<td>5/5</td>
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<tr>
<td>Colon</td>
<td>0/4*</td>
</tr>
<tr>
<td>Stomach</td>
<td>0/3</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0/3</td>
</tr>
<tr>
<td>Liver</td>
<td>2/2</td>
</tr>
<tr>
<td>Pancreas</td>
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</tr>
<tr>
<td>Exocrine</td>
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</tr>
<tr>
<td>Endocrine</td>
<td>0/3</td>
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<tr>
<td>Kidney</td>
<td></td>
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<tr>
<td>Tubules</td>
<td>2/2</td>
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<tr>
<td>Glomeruli</td>
<td>0/2</td>
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<tr>
<td>Prostate</td>
<td>3/3</td>
</tr>
<tr>
<td>Bladder</td>
<td>4/4</td>
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<tr>
<td>Lymph node</td>
<td>0/1</td>
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<tr>
<td>Tonsil</td>
<td>0/1</td>
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<tr>
<td>Spleen</td>
<td>0/1</td>
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<tr>
<td>Brain</td>
<td>0/2</td>
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<tr>
<td>Spinal cord</td>
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<tr>
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<tr>
<td>Thyroid</td>
<td>0/1</td>
</tr>
<tr>
<td>Skin</td>
<td>1/1</td>
</tr>
</tbody>
</table>

* Equivocal or trace staining was observed in some specimens and may reflect weak expression of the EA1 antigen.

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**Fig. 2.** Representative EA1 staining of human lung carcinomas. EA1 reactivity with lung epidermoid carcinoma (A) and lung adenocarcinoma (B). C, absence of EA1 staining with SCLC.
corresponding neoplasms represent a differentiation continuum (25). In this regard, it would be interesting to evaluate the expression of the EA1 antigen in relation to the loss of SCLC and the acquisition of LCLC characteristics by these cell lines.

In conclusion, studies examining the expression of EA1 and other MoAb-defined antigens by fetal, adult, and neoplastic lung tissues should enhance our knowledge of normal lung differentiation and may advance our understanding of the histogenesis of pulmonary neoplasms. More extensive correlation of MoAb-defined phenotypes with clinical features will be required to delineate the role of MoAb analyses in identifying clinically significant subtypes of human lung carcinomas. The results of these investigations may refine our ability to classify this complex group of related neoplasms.

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REFERENCES

MoAb TO A HUMAN NON-SMALL CELL LUNG CARCINOMA ANTIGEN


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