Isolation of Small Cell Lung Cancer-associated Antigen from Human Brain

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ABSTRACT

Previous studies have demonstrated that monoclonal antibody TFS-4 recognizes a cell surface antigen with a molecular weight of 124,000 expressed selectively on small-cell lung cancer but not on non-small-cell lung cancers and that it cross-reacts with human brain. The antigen determinant on small-cell lung cancer and that on brain shared common characteristics, i.e., trypsin sensitivity, heat lability, and neuraminidase resistance, suggesting that they are similar peptides (T. Okabe et al., Cancer Res., 44: 5273–5278, 1984; J-i. Watanabe et al., Cancer Res., 47: 826–829, 1987). In order to elucidate the nature of this unique antigen recognized by TFS-4, we have purified the antigen to homogeneity from human brain. The antigen was solubilized from brain with 0.5% Nonidet P-40, precipitated with 50% ammonium sulfate, and subsequently purified by sequential chromatographies, i.e., diethylaminoethyl-Sepharose P-40, precipitated with 50% ammonium sulfate on ice and then bound components were eluted in fractions of 8 ml with the

INTRODUCTION

We have previously reported the production of four monoclonal antibodies, TFS-1-4, raised against SCLC. TFS-4 has been shown to react selectively with SCLC but not with squamous cell or adenocarcinoma of the lung. It recognized carcinoid tumors and neuroblastoma but not cancers from other organs (1, 2). The immunoprecipitation study of the antigen on SCLC revealed a molecular weight of 124,000 on SDS-PAGE in reducing conditions (1). In normal tissues TFS-4 cross-reacted with central nervous tissues. Characterization of antigens on human brain and SCLC demonstrated that they shared common features; they were trypsin sensitive and heat labile but neuraminidase resistant (2). Since the investigation of the antigen which is expressed selectively in SCLC may be of great importance in the understanding of this cancer, purification studies were carried out using human brain tissues as the source of antigen.

In this paper, we describe the isolation of the antigen from human cerebral.

MATERIALS AND METHODS

Monoclonal Antibody. The production of TFS-4 hybridoma has been reported in detail (1). The antibody was produced either as culture supernatants or as ascites in BALB/c mice. TFS-4 hybridoma cells were cultured for 3 days at a concentration of 1 x 10^6 cells/ml in RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% fetal calf serum (Flow). Conditioned medium was centrifuged at 1000 x g for 10 min, and supernatant was frozen until use. BALB/c mice were intraperitoneally injected with 5 x 10^6 TFS-4 hybridoma cells. Ascites was collected after 10 days and centrifuged at 1000 x g for 20 min to eliminate cell debris. The supernatant was precipitated with 50% ammonium sulfate on ice and then bound components were eluted in fractions of 8 ml with the

Ammonium Sulfate Precipitation. Preliminary examination revealed that 50% ammonium sulfate precipitated most of the antigenic reactivity (data not shown). Ammonium sulfate powder was added to the supernatant with constant stirring until a concentration of 50% was reached. Stirring was continued for 10 min at 4°C, and the mixture was allowed to stand for 30 min. The precipitated proteins were recovered by centrifugation at 20,000 x g for 20 min and dissolved in 23 ml of Tris-HCl (pH 7.4) containing 2 mm phenylmethylsulfonyl fluoride, 0.01% EDTA, and 0.02% NaN₃. Homogenate was centrifuged at 10,000 x g for 20 min, and the membrane fraction was recovered as supernatant. To solubilize antigen from cell membranes, NP-40 was added to the supernatant up to 0.5% while stirring at 4°C for 30 min. The mixture was centrifuged at 100,000 × g for 60 min, and the supernatant was subjected to ammonium sulfate precipitation (4).

DEAE-Sepharose Ion Exchange Chromatography. The fraction precipitated by ammonium sulfate was applied to a DEAE-Sepharose CL-6B column (× 8 cm) previously equilibrated with Tris buffer. The column was washed with 300 ml of Tris buffer containing 50 mm NaCl, and then bound components were eluted in fractions of 8 ml with the use of a linear gradient of 50 to 300 mm NaCl in the same buffer at a flow rate of 80 ml/h. Fractions were assayed for antigen reactivity with TFS-4 by immunoblotting, and the active fractions were concentrated with Amicon ultrafiltration using a YM10 membrane.

Immunoblotting. Anti-mouse IgG (Vector) (1:2000 in 1% gelatin in PBS) was incubated at room temperature for 30 min. The membranes were pretreated with 1% gelatin in PBS for 20 min. Then, they were reacted with TFS-4 antibody for 1 h at room temperature. After three washes with PBS, membranes were exposed to biotinylated anti-mouse IgG (Vector, Burlingame, CA) (1:2000 in 1% gelatin in PBS) for 30 min, washed 3 times, and then incubated with avidin D:peroxidase (Vector) (1:2000 in 1% gelatin in PBS). Finally the membranes were reacted with HRP reagent (Bio-Rad) according to the instructions of the manufacturer. The dilution of the samples to give the same intensity of the reaction of control sample was designated as the titer of the antigen. One unit was defined as the antigenic activity of the undiluted NP-40-solubilized fraction.

Protein Assay. Protein concentration was assayed with the method of Lowry et al. (3) using bovine serum albumin as a standard unless otherwise described.

Homogenization and Solubilization of Antigen from Human Brain. Human brain was obtained at autopsy and frozen at −70°C within 2 h of death. Fifty g of cerebrum were homogenized with Polytron mixer in the presence of 150 ml of PBS containing 0.25 m sucrose, 2 mm phenylmethylsulfonyl fluoride, 0.01% EDTA, and 0.02% NaN₃. Homogenate was centrifuged at 10,000 x g for 20 min, and the membrane fraction was recovered as supernatant. To solubilize antigen from cell membranes, NP-40 was added to the supernatant up to 0.5% while stirring at 4°C for 30 min. The mixture was centrifuged at 100,000 × g for 60 min, and the supernatant was subjected to ammonium sulfate precipitation (4).

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1 To whom requests for reprints should be addressed.

1 The abbreviations used are: SCLC, small-cell lung cancer; PBS, phosphate-buffered saline; NP-40, Nonidet P-40; APUD, amine precursor uptake, decarboxylase; HPLC, high-pressure liquid chromatography; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; BASCA, brain-associated small-cell lung cancer antigen.

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Table 1  Purification of BASCA from human brain

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>140</td>
<td>7.7</td>
<td>1080</td>
<td>32</td>
<td>4.2</td>
<td>4480</td>
<td></td>
</tr>
<tr>
<td>NP-40</td>
<td>130</td>
<td>7.7</td>
<td>1000</td>
<td>32</td>
<td>4.0</td>
<td>7400</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>23</td>
<td>8.0</td>
<td>184</td>
<td>32</td>
<td>8.0</td>
<td>450</td>
<td>61</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>56</td>
<td>1.0</td>
<td>56</td>
<td>8</td>
<td>8.0</td>
<td>260</td>
<td>35</td>
</tr>
<tr>
<td>Anti-TFS-4</td>
<td>4</td>
<td>0.053</td>
<td>0.212</td>
<td>64</td>
<td>1200</td>
<td>4000</td>
<td>64</td>
</tr>
<tr>
<td>HPLC</td>
<td>1</td>
<td>0.016</td>
<td>0.016</td>
<td>64</td>
<td>4000</td>
<td>64</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* Since NP-40 interfered with adsorption of antigen onto nitrocellulose paper, the activity in this fraction was weakest. The reaction of the undiluted sample of this fraction was defined as 1 unit. It was apparently an underestimation, and therefore, specific activity and total activity were not shown.

† Protein concentration was assessed by absorbance at 280 nm.

RESULTS

TFS-4 antigen was successfully solubilized from the cell membrane fraction of human brain with 0.5% NP-40. Comparing the anti-TFS-4 activity in homogenate and that recovered in ammonium sulfate precipitates, some 16% antigen was solubilized with NP-40 (Table 1). The concentration of the antigen in the NP-40-solubilized fraction was not reliably assessed by immunoblotting, because the detergent interfered with adsorption of protein onto nitrocellulose membrane. Fifty % ammonium sulfate precipitates are considered to contain most of the antigenic activity, since trace activity was detected in the supernatant. Lipids included in brain homogenate were separated in this step, as they formed a layer floating on ammonium sulfate precipitates are considered to contain most of the antigenic activity, since trace activity was detected in the supernatant. Lipids included in brain homogenate were separated in this step, as they formed a layer floating on ammonium sulfate precipitates.

Fig. 1. DEAE-Sepharose chromatography of TFS-4 antigen. The ammonium sulfate-precipitated proteins were applied to a column (4 x 8 cm) of DEAE-Sepharose CL-6B equilibrated with 100 mM Tris buffer (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride, 0.01% EDTA, 0.005% NP-40, and 0.02% NaN₃. Elution was performed with a linear gradient of 50 to 500 mM NaCl in Tris buffer. The antigenic reactivity was detected by immunoblotting using TFS-4 as a primary antibody. —— A300, — — conductivity; O, antigenic reactivity.

Fig. 2. Immunoaffinity chromatography of TFS-4 antigen. Proteins eluted from a DEAE-Sepharose CL-6B column were concentrated with Amicon ultrafiltration using TFS-4 antibody (1 x 3 cm). After extensive washing with Tris buffer, the antigen was eluted with 100 mM glycine-HCl (pH 2.5). The fractions containing proteins detected with A300 were pooled, immediately dialyzed against 10 mM P, buffer, assayed for antigenic reactivity, and lyophilized. —— A300.

Fig. 3. Gel permeation HPLC of TFS-4 antigen. Lyophilized material from immunoaffinity chromatography was resolved in 100 μl of distilled water and applied to HPLC with a TSK G4000SW column (0.75 x 60 cm) equilibrated with PBS. Elution was carried out at a flow rate of 0.5 ml/min, and each fraction was collected in 0.5 ml. Each fraction was assessed for antigenic reactivity by immunoblotting. SDS-PAGE Analysis of Purified Antigen. The active fraction from gel permeation HPLC was analyzed on SDS-PAGE (T = 7.5%) under reducing conditions (2.5% mercaptoethanol) (5). Protein was detected using silver staining (Daiichi Kagaku, Tokyo, Japan). Molecular weight markers included myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin (Bio-Rad).
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fig. 4. Analysis of purified antigen from human brain on SDS-PAGE detected by silver staining. Purified antigen (0.16 ng) was applied to SDS-PAGE (T = 7.5%) under reducing conditions (2.5% mercaptoethanol). The gel was stained with a silver staining technique. Molecular weight markers included myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin (0.2 ng each).

antigen was bound to an immunoaffinity column and eluted with glycine-HCl buffer (pH 2.5) (Fig. 2). The fractions containing proteins were concentrated by lyophilization and applied to a TSK G4000SW column. Gel permeation HPLC revealed three major peaks (Fig. 3). Only the first peak fraction showed antigenic reactivity. The purified protein from gel permeation HPLC showed a single protein band on SDS-PAGE by a silver staining technique. The molecular weight was estimated at 124,000 (Fig. 4). In an overall purification procedure, we achieved a 1000-fold increase in specific activity with an 8.6% antigen recovery.

DISCUSSION

Small-cell lung cancer-associated antigen was purified to apparent homogeneity from human brain tissues by sequential ion exchange chromatography, immunoaffinity chromatography, and gel permeation HPLC. The purified antigen showed a single protein band with a molecular weight of 124,000 on SDS-PAGE under reducing conditions.

Previous studies have demonstrated that the antigen on SCLC has a molecular weight of 124,000 on SDS-PAGE under reducing conditions (1). These observations support the idea that the antigen on SCLC and that on brain represent structurally related peptide(s).

Biochemical properties of SCLC have been extensively investigated. Pearse identified a widely distributed system of cells having APUD properties (6). SCLC tumors have been shown to express these APUD cell properties (7). In contrast to the well-investigated biochemical characteristics of SCLC, our knowledge of surface antigens specifically expressed on SCLC cells is pitifully meager. TFS-4 monoclonal antibody recognized a unique antigen(s) that was selectively expressed on SCLC and neuronal tissues (BASCA). The present study described the isolation of BASCA. Although SCLC cells expressed many of the APUD cell properties, it has not been known if SCLC cells arise from neural crest or endodermal structure as do other bronchogenic carcinomas. BASCA was expressed on carcinoid tumors, neuroblastoma, and retinoblastoma (2). In normal tissues, BASCA was detected in neuronal tissues, cardiac muscle, and some endocrine cells. These observations suggest that SCLC specifically expresses the antigen common to brain and endocrine tissues.

The antigen isolated from human brain should aid in the study of the nature and origin of SCLC.

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