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Isolation of Small Cell Lung Cancer-associated Antigen from Human Brain[1]

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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 antigenic 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 ion exchange, immunoaffinity, and gel permeation high-pressure liquid chromatography. The antigenic reactivity was assessed by immunoblotting using TFS-4 as a primary antibody. The purified antigen showed a single protein band with a molecular weight of 124,000 on sodium dodecylsulfate-polyacrylamide gel electrophoresis detected by a silver staining technique. The results suggest that the antigen on brain tissues is structurally related to the molecule expressed on small-cell lung cancer.

INTRODUCTION

We have previously reported the production of four monoclonal antibodies, TFS-1-4, raised against SCLC.[3] 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 cerebrum.

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 1×10^5 cells/ml in RPMI 1640 medium (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% fetal calf serum (Flow). Conditioned medium was centrifuged at $1000 \times g$ for 10 min, and supernatant was frozen until use. BALB/c mice pretreated with 0.5 ml of Pristane were given injections i.p. of 1×10^7 TFS-4 hybridoma cells. Ascites was collected after 10 days and centrifuged at $1000 \times g$ for 20 min to eliminate cell debris. The supernatant was precipitated with 50% ammonium sulfate on ice and clarified by centrifugation at $20,000 \times g$ for 20 min. The precipitate was dissolved in 20 mM Tris-HCl (pH 7.8) with 40 mM NaCl (Buffer A) and dialyzed against 20 mM Tris-HCl buffer with 20 mM NaCl (Buffer B). The dialyzed sample was applied to a DE-52 column equilibrated with Buffer B and eluted with a gradient of 20 to 200 mM NaCl. Fractions were assessed for IgG content by immunoblotting, and the peak fraction was concentrated with Amicon ultrafiltration using a YM10 membrane.

Determination of Antigen Titer. Two µl of serially diluted samples were blotted onto nitrocellulose membrane (Bio-Rad, Richmond, CA) and air dried. To prevent nonspecific binding of antibodies, 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% NaN_3. Homogenate was centrifuged at $10,000 \times g$ 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 $100,000 \times g$ for 60 min, and the supernatant was subjected to ammonium sulfate precipitation (4).

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 $20,000 \times g$ for 20 min and dissolved in 23 ml of 100 mM Tris-HCl (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride, 0.01% EDTA, 0.005% NP-40, and 0.02% NaN_3 (Tris buffer). The suspension was then dialyzed against four changes of Tris buffer.

DEAE-Sepharose Ion Exchange Chromatography. The fraction precipitated by ammonium sulfate was applied to a DEAE-Sepharose CL-6B column (4 × 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 500 mM NaCl in the same buffer at a flow rate of 80 ml/h. Fractions were assessed for antigen reactivity with TFS-4 by immunoblotting, and the active fractions were concentrated with Amicon ultrafiltration using a YM100 membrane.

Immunoaffinity Chromatography. TFS-4 monoclonal antibody pro-

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[3] 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*

Step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Total activity (units)	Recovery (%)
Homogenate	140	7.7	1080	32	4.2	4480	
NP-40	130	7.7	1000	(Unity[a])			
Ammonium sulfate	23	8.0	184	32	4.0	740	100
DEAE-Sepharose	56	1.0	56	8	8.0	450	61
Anti-TFS-4	4	0.053	0.212	64	1200	260	35
HPLC	1	0.016[b]	0.016	64	4000	64	8.6

[a] 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.
[b] Protein concentration was assessed by absorbance at 280 nm.

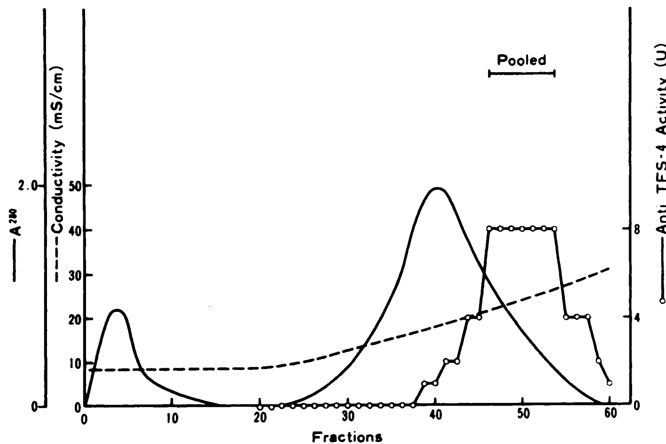

Fig. 1. DEAE-Sepharose chromatography of TFS-4 antigen. The ammonium sulfate-precipitated proteins were applied to a column (4 × 8 cm) of DEAE-Sepharose CL-6B equilibrated with 100 mM Tris buffer (pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride, 0.01% EDTA, 0.005% NP-40, and 0.02% NaN_3. Elution 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. ——, A_{280}; - - -, conductivity; O, antigenic reactivity.

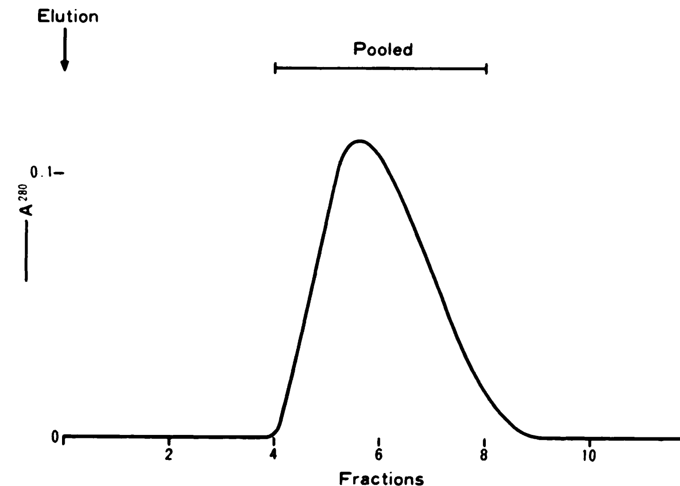

Fig. 2. Immunoaffinity chromatography of TFS-4 antigen. Proteins eluted from a DEAE-Sepharose CL-6B column were concentrated with Amicon ultrafiltration using a YM100 membrane and applied to Sepharose 4B column coupled with TFS-4 antibody (1 × 3 cm). After extensive washing with Tris buffer, the antigen was eluted with 100 mM glycine-HCl (pH 2.5). The fractions were collected in 1 ml, and those containing proteins detected with A_{280} were pooled. They were immediately dialyzed against 10 mM P_i buffer, assayed for antigenic reactivity, and lyophilized. ——, A_{280}.

duced as ascites in BALB/c mice was purified by ammonium sulfate precipitation and DE-52 ion exchange chromatography as described above. Five mg of purified immunoglobulin were coupled with 1 ml of CNBr-activated Sepharose 4B according to the instructions of the manufacturer. Concentrated fractions containing TFS-4 antigenic activity were applied to Sepharose 4B coupled with TFS-4 antibody (1 × 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 A_{280} were pooled, immediately dialyzed against 10 mM P_i buffer, assayed for antigenic reactivity, and lyophilized.

Gel Permeation HPLC. Lyophilized material from immunoaffinity chromatography was resolved in 100 µl of distilled water and applied to HPLC with a TSK G4000SW column (0.75 × 60 cm) (Toyo Soda, Tokyo, Japan) equilibrated with PBS. Elution was carried out with the same buffer 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 by silver staining (Daiichi Kagaku, Tokyo, Japan). Molecular weight markers included myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin (Bio-Rad).

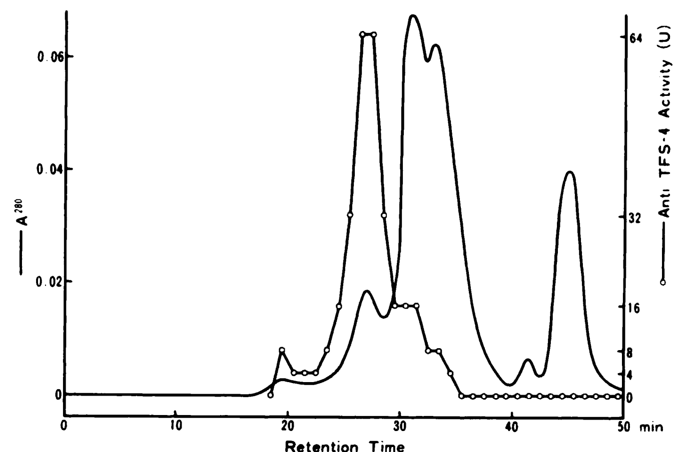

Fig. 3. Gel permeation HPLC of TFS-4 antigen. Lyophilized material from an immunoaffinity column was resolved in 100 µl of distilled water and applied to HPLC with a TSK G4000SW column (0.75 × 60 cm) equilibrated with PBS. Elution was carried out with PBS at a flow rate of 0.5 ml/min, and each fraction was collected in 0.5 ml. Antigenic reactivity was detected by immunoblotting. ——, A_{280}; O, antigenic reactivity.

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 solution after centrifugation. Sixty % of the antigen applied to the DEAE-Sepharose CL-6B column was eluted in fractions containing 150 to 300 mM NaCl in Tris Buffer (Fig. 1). TFS-4

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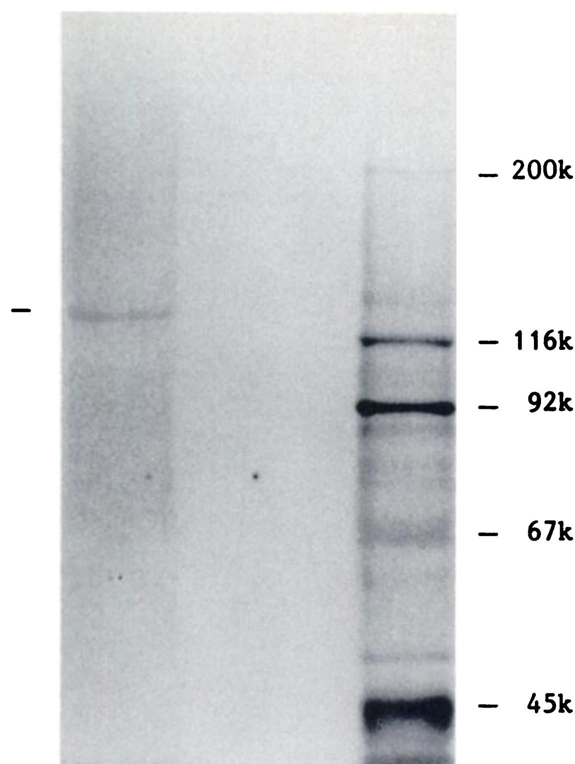

Fig. 4. Analysis of purified antigen from human brain on SDS-PAGE detected by silver staining. Purified antigen (0.16 μg) was applied to SDS-PAGE (T = 7.5%) under reducing conditions (2.5% mercaptoethanol). The gel was stained with a silver staining technique. Molecular weight markers included myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin (0.2 μg 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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