Characterization of neuroectodermal antigen by a monoclonal antibody and its application in CSF diagnosis of human glioma

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Monoclonal antibodies were produced by immunization of the human glioma cell line SK-MG-4. One of the antibodies, designated G-22, reacted with 18 of 20 glioma cell lines, two melanoma cell lines, and three lung cancer cell lines, but not with 39 cell lines derived from sarcoma, carcinoma, or hematopoietic tumors. The antigen was expressed in the brain of human fetuses in early gestation (9 weeks) but not in late gestation (8 months) or in normal adult brain, suggesting that the antibody recognizes neural differentiation antigens expressed by neuroectodermal origin. A high incidence of positive antigens has been observed in gliomas but not in the other neural tumors, such as ependymomas, meningiomas, and neuroblastomas. Thus, the antigen defined by the G-22 monoclonal antibody could be defined as glioma-associated antigen. Pulse-labeling with tritiated leucine and subsequent immunoprecipitation of the solubilized cell membrane revealed that the antigen recognized by this antibody had a molecular weight of 67 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

It was shown by dot-blot enzyme-linked immunospecific assay (ELISA) that the antigen could be detected in the cerebrospinal fluid (CSF) from patients with gliomas. From analysis of affinity chromatography and SDS-PAGE, the antigen present in the CSF had a molecular weight similar to that of a 1% Nonidet P-40 (NP-40) extract from a glioma cell line. When the antigen in CSF was quantitatively assayed by ELISA, the mean antigen level (expressed as optical density at 450 nm) in the CSF of seven patients was 0.8 ± 0.28 (mean ± standard deviation), which was significantly higher than the 0.38 ± 0.14 level observed in the CSF of 15 patients with nonglioma brain tumors and the 0.23 ± 0.09 level in the CSF of four patients without brain tumors. These results indicate that the monoclonal antibody G-22 is useful for the diagnosis of glioma.

KEY WORDS • monoclonal antibody • glioma • cerebrospinal fluid • brain neoplasm

MONOCLONAL antibodies cultured by the hybridoma technique have proved useful in identifying and characterizing antigens present on human neoplastic cells. We have produced many monoclonal antibodies by immunization of various human glioma cell lines. These antibodies have been shown to react with a variety of cell lines of neuroectodermal origin. The reactivity spectrum of these antibodies has been defined in previous communications.26,27

In the present investigation, the serological and biochemical characteristics of a glioma-associated antigen were defined by an anti-glioma monoclonal antibody designated as G-22. A quantitative assay of antigen shedding in the cerebrospinal fluid (CSF) of glioma patients was developed and the assay was evaluated for its clinical usefulness.

Materials and Methods

Tissue Culture

The cell lines used in this study are summarized in Table 1. The GL and MK cell lines were established in our laboratories from surgically excised malignant glioma specimens. The KNS-42 and KMS-II cell lines were established at Kyushu University and Kumamoto University, Japan, respectively. The origin of other cell
TABLE 1
Reactivity of G-22 with 64 cell lines*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Reactivity</th>
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<tbody>
<tr>
<td>neural tumors</td>
<td>glioma</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>carcinomas (cont)</td>
<td>lung</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
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<tr>
<td>+</td>
<td>+</td>
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* Reactivity of G-22 was tested by immune adherence rosette assay and by the indirect immunofluorescence technique, which gave essentially the same results. + = positive reaction with G-22 and - = negative reaction with G-22.

Solubilization of Antigen from Cloned Cells or Tissue Specimens Using NP-40

The cells were grown to confluence in a 25-sq cm Falcon flask, harvested with a rubber policeman, and suspended in a solution of 0.15 M NaCl and 10 mM phosphate-buffered saline (PBS), pH 7.2, containing 0.1 mM phenyl-methyl-sulfonylfluoride (PMSF). After centrifugation, 0.5 ml of PBS-PMSF containing 1% Nonidet P-40 (NP-40) was added to the cell pellet containing 1 to 10 × 10^7 cells. The mixture was agitated for 15 minutes at 0°C and the insoluble residue was removed by centrifugation at 10,000 G for 5 minutes at room temperature. The NP-40 extract was collected and stored at −80°C until use.

Normal tissues from human adult and fetal brain were obtained at autopsy, and brain-tumor tissues were harvested at surgery. The tissues were immediately frozen in dry ice and stored at −80°C until use. The tissue samples were cut into a 0.5-cm diameter block, washed three times with PBS, and homogenized in PBS-PMSF containing 1% NP-40. After centrifugation at 10,000 G for 15 minutes, the supernatant was kept frozen at −80°C. Determination of the protein concentration in the supernatant was performed as described by Hess, et al.*

Sources of Cerebrospinal Fluid

Samples of CSF were obtained either by lumbar puncture or by puncture of a subcutaneous Ommaya reservoir connected to the ventricular system of patients. Twenty-six samples of CSF were obtained from patients with neurological diseases: seven patients with glioblastoma multiforme or anaplastic astrocytoma diagnosed histopathologically and/or clinically, 15 with nongliomatous intracranial tumors (such as meningioma, neurinoma, lymphoma, and metastatic tumor from the rectum), and four non-neoplastic diseases (such as subarachnoid hemorrhage, ossification of the posterior longitudinal ligament, and normal-pressure hydrocephalus).

* Most cell lines not established in Japan were obtained from the Memorial Sloan-Kettering Cancer Center, New York, New York.
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Procedures for Dot-Blot ELISA

For the detection of antigen by G-22, a three-step dot-blot ELISA was performed. As the first step, NP-40 extracts or CSF samples were spotted onto nitrocellulose membrane (pore size 0.45 μm) using a minifold apparatus. The membrane was washed three times with 0.05% vol/vol Tween 20 and 5% skim milk in PBS and gently agitated for 2 hours in the same solution containing G-22 at room temperature. Binding of the antibody was detected in the second step as follows. After several washes in PBS containing 0.05% Tween 20 for 30 minutes, the membrane was incubated for 1 hour at room temperature with peroxidase-coupled goat antibodies against mouse immunoglobulins (KPL) diluted 1:1000 in the same buffer; it was then washed several times in PBS alone, and was cut into fragments each containing a spot. The third step was to quantitate the amount of antibody bound to the membrane. The fragments were placed individually into the wells of Falcon microtiter plates and again washed with PBS, then soaked for 15 minutes in the enzyme substrate containing O-phenylenediamine (0.5 mg/ml) and 0.01% H2O2 in 0.1 M citrate-phosphate buffer, pH 5.0, to detect peroxidase activity. After the membrane fragments were removed from the solution, 4 M H2SO4 was added to them to stop the reaction. The optical density at 450 nm was immediately determined.

Radiolabeling, Immunoprecipitation, and Gel Electrophoresis

After preincubation in leucine-free Eagle’s MEM, the glioma cell line SK-MG-4 was labeled with tritiated (3H)-leucine for 1 to 6 hours at 37°C. The labeled cells were solubilized in PBS-PMSF containing 1% NP-40. The extracts containing 480 mg protein were preincubated with Pansorbin for 5 minutes at room temperature to remove nonspecifically absorbed materials. After centrifugation, the supernatant was incubated with 100 μl of mouse monoclonal antibody G-22, which contained about 0.8 mg of immunoglobulin G (IgG). Immune complexes were isolated by Pansorbin, and then denatured by incubation for 5 minutes with buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% 2-mercaptoethanol, and 10% glycerol at 95°C. The immunoprecipitated materials were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Jones. After electrophoresis, fluoroscopy was carried out with EN3HANCE as specified by the manufacturer.

Purification of Antigen from Glioma Cell Line or CSF

The IgG fraction from the monoclonal antibody G-22 ascites was purified with a protein A monoclonal antibody purification system kit according to the manufacturer’s directions. The purified IgG was used for the subsequent conjugation to CNBr-activated sepharose. Extensive screening of other cell lines, including ependymoma, neuroblastoma, hepatoma, renal cancer, colon cancer, and lymphoma, revealed no reactivity with G-22.

Serological Specificity of G-22

The monoclonal antibody G-22 had a γ2a chain. The serological specificity of G-22 was tested by two different assays, immune adherence and indirect immunofluorescence, on a panel of 64 human tumor cell lines. Some of these results are summarized in Table 1. The G-22 monoclonal antibody reacted with 18 of 20 glioma cell lines, and also with two melanoma cell lines and three lung cancer cell lines. Extensive screening of other cell lines, including ependymoma, neuroblastoma, hepatoma, renal cancer, colon cancer, and lymphoma, revealed no reactivity with G-22.

Characterization of G-22-Defined Antigen by Pulse-Labeling

For the characterization of G-22-defined antigen, SK-MG-4 cells were pulse-labeled for either 1 or 6 hours after solubilization of the cells, and the material precipitated with G-22 antibody was analyzed by SDS-PAGE. A parallel experiment was performed using KMS-II cells which did not react with G-22. A single band of about 67 kD was demonstrated in the SK-MG-4 cell lines incubated for 1 hour with 3H-leucine, and the band became denser in the cells incubated for 6 hours (Fig. 1). This band was not detectable in the G-22-negative cell line (KMS-II) even when incubated for 6 hours.

Purification of G-22-Defined Antigen from NP-40 Extracts

About 1.2 ml (9.8 mg protein) of NP-40 extracts from SK-MG-4 was applied on a column of sepharose 4B coupled with G-22. The column was extensively washed and then eluted with the sodium acetate buffer (pH 3). As shown in Fig. 2, purification with the immunoadsorbent column was tremendously effective; most of the protein in the NP-40 extracts emerged in the flow-through fraction without being absorbed. How-
FIG. 1. Characterization of the G-22-defined antigen in SK-MG-4 cells. Both SK-MG-4 (a glioma cell line with positive reaction with G-22) and KMS-II (an ependymoma cell line with negative reaction with G-22) were labeled with 3H-leucine for 1 hour or 6 hours. After preparation of the cell extract with NP-40, the antigen was precipitated with the G-22 monoclonal antibody. The immunoprecipitate was analyzed by SDS-PAGE (see text). A protein band (arrow) of 67 kD molecular weight (M.W.) was observed only in the materials immunoprecipitated with G-22.

However, most of the antigenic activity was eluted by the sodium acetate buffer. When this purified material was analyzed with SDS-PAGE, a single 67-kD band was clearly visualized. This result correlated well with the results of the pulse-labeling experiment (Fig. 3, arrow). When CSF samples from patients with glioma were similarly analyzed, a 67-kD band was observed. However, CSF samples obtained from patients without intracranial tumors did not show a band.

Dot-Blot ELISA

To evaluate the specificity and the validity of the assay system, different amounts of NP-40 extracts from various glioma cell lines and from nonglioma cell lines were spotted on nitrocellulose membrane, and an ELISA was performed. The concentration of G-22-defined antigen in the samples could be determined by a standard curve using NP-40 extract from SK-MG-4 cells, which is essentially linear from about 0.1 to 8 mg solubilized protein/ml (Fig. 4). On the other hand, the antigen level in the KMS-II extract remained very low even in the presence of 8 mg solubilized protein. The same study was performed with several other cell lines, showing that dot-blot ELISA reflected the serological specificity. It should be noted that the antibody did not react with normal adult brain nor with fetal brain of 8 months' gestation, whereas good reactivity was demonstrated with fetal brain of 9 weeks' gestation. In addition, G-22 did not react with the NP-40 extracts from rat fetal or adult brain or with rat glioma cell lines (Fig. 5).

Dot-Blot ELISA for Diagnostic Use

Samples of CSF were obtained from three groups of patients: those with glioma, those with nongliomatous intracranial tumor, and those with intracranial nonneoplastic diseases. For the dot-blot ELISA, 100 μl of each CSF sample was used. As shown in Fig. 6, the mean antigen levels in CSF from patients with nongliomatous and nonneoplastic diseases (expressed as the optical density at 450 nm) were 0.38 ± 0.14 and 0.23 ± 0.09, respectively. The mean antigen levels in CSF from glioma patients was 0.8 ± 0.28, which was significantly higher than those from patients with nongliomatous and nonneoplastic diseases.
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FIG. 3. Characterization of the G-22-defined antigen purified by immunoabsorbent chromatography. The material purified by immunoabsorbent column as shown in Fig. 2 was analyzed by SDS-PAGE (see text). To reveal the protein band (arrow) the gel was stained with silver. Lane 1: marker proteins; Lane 2: samples eluted by sodium acetate buffer, pH 3.0; Lane 3: original NP-40 extract from SK-MG-4; Lane 4: flow-through fraction.

higher than the value in CSF from patients with non-gliomatous brain tumor (p < 0.01) or patients without brain tumor (p < 0.001).

Discussion

Differentiation antigens, which are selectively expressed on the surface or in the cytoplasm of normal cells according to their lineage or stage of development, were demonstrated on the surface of neoplastic cells of the same lineage. Several differentiation antigens defined in human glioma have also been detected by studies with conventional or monoclonal antibodies and reported as brain-specific proteins found in the cytoplasm (such as glial fibrillary acidic protein (GFAP), S-100 protein, or neuron-specific enolase), and neuroectodermal or neurohematopoietic antigens found on the cell surface.

The antigen identified with the monoclonal antibody G-22 could be recognized as a neuroectodermal antigen from the data presented here. The antigen was expressed on the surface of neoplastic cells established from glioma, melanoma, and lung cancer. It has been reported that lung cancer cells, especially the small cell type, express neuron-specific enolase in the cytoplasm and are considered to relate with neuroectoderm. The antigen defined by G-22 was also expressed on specific normal cells such as fetal brain cells of early gestational age.

Biochemically, the antigen was proved to be a membrane molecule on human glioma cells with a molecular weight of 67 kD. The presence of this antigen was confirmed not only in NP-40 extracts from glioma cells but also in CSF samples obtained from patients with gliomas by immunoabsorbent chromatography and SDS-PAGE. Similar biochemical analysis of the neuroectodermal antigens was reported by a few groups of investigators. Their spectrum of reactivity and biochemical nature were, however, different from our G-22-reactive antigen. The monoclonal antibody 81C6 defined a 230-kD polypeptide which was present in glioma mesenchymal extracellular matrix antigen and was expressed in cultured glioma and fibroblast cells. An antigen defined by the UJ171-11 monoclonal antibody was expressed in neuroblastoma, medulloblastoma, ganglioglioma, and melanoma cell lines but not in glioma cell lines. The molecule was a glycoprotein of 220 to 240 kD. An antigen defined by the R24 monoclonal antibody was detected on the cell surfaces of melanoma and glioma cells and identified as a GD3 ganglioside.

If an antibody can detect an antigen that is expressed predominantly or exclusively on a certain type of neoplastic cells and is shed into the CSF circulation or serum, it may be possible to establish a screening test for tumor diagnosis by identifying the antigen in the serum or CSF. For example, radioimmunoassay with a
monoclonal antibody (CA19-9) has been utilized to detect a monosialo-ganglioside from colorectal tumor cells in the serum of afflicted patients. There are no reports of diagnosis of brain tumor using serum or CSF samples; however, some marker substances in the CSF were confirmed to be of diagnostic value. The levels of desmosterol, polyamine, and GFAP are reported to be elevated in the CSF of patients with brain tumors, and determination of the levels of these marker substances in CSF was shown to be useful as an indicator of tumor progression. However, those markers lack tumor specificity and their level must be extremely high to be of any significance in the screening of patients.

On the other hand, the neuroectodermal differentiation antigen recognized by the G-22 monoclonal antibody is a glioma-associated antigen without expression on nongliomatous brain tumor or on normal adult brain cells, and its shedding can be demonstrated in the CSF, indicating its potential usefulness in the diagnosis of glioma. Indeed, quantitative assay of the antigen in CSF with dot-blot ELISA could be used to differentiate patients with glioma from patients with no intracranial tumors and also from patients with nongliomatous brain tumors. Antigen levels (as defined by optic density at 450 nm) were lower than 0.5 (mean level 0.23) in the CSF of patients without brain tumor while they were higher than 0.5 (mean level 0.80) in all the patients with glioma. The CSF from only 20% of patients (three of 15) with nongliomatous brain tumor exhibited antigen levels higher than 0.5 (mean level 0.38).

References
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