Antigen related to cell proliferation in malignant gliomas recognized by a human monoclonal antibody

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A human monoclonal antibody (CLN-IgG) was produced from a human-human hybridoma derived from lymphocytes of a patient with cervical carcinoma. The reactivities of this antibody with various human glioma tissues and cultured glioma cells and the characterization of the antigen recognized by CLN-IgG on malignant glioma cells were analyzed and reported. CLN-IgG reacted with various human glioma cells and glioma tissues, especially glioblastoma, but did not react with normal brain tissues or fetal brain tissues. A large amount of antigen recognized by CLN-IgG was expressed on cell membranes of undifferentiated glioma cells and of glioma cells at the G2/M tumor growth phase in cycling cells. Antigen recognized by CLN-IgG was detected in only one of seven samples of cyst fluid, and was not detected in 27 serum samples or 18 samples of cerebrospinal fluid from glioma patients. CLN-IgG exhibited antibody-dependent cell cytotoxicity against U-251MG glioma cells and primary cultured cells of glioblastomas and anaplastic astrocytomas. These data suggest that the antigen recognized by CLN-IgG might be related to cell proliferation in malignant gliomas. Thus, CLN-IgG might be useful for immunotherapy or immunoimaging of malignant gliomas.

KEY WORDS  •  brain tumor  •  immunotherapy  •  glioma  •  monoclonal antibody

Since the development of hybridoma technology 15 years ago, a number of murine monoclonal antibodies against human cancer cells have been produced and many antigens have been identified. The clinical use of murine monoclonal antibodies produced by mouse-mouse or mouse-human hybridomas in the treatment of human cancers has inspired new concepts for cancer diagnosis and treatment. However, there are some practical problems associated with the clinical use of foreign antibodies in humans, such as the induction of the human anti-mouse response, possibly reducing the efficacy of the treatment.

Several human monoclonal antibodies against human cancer cells have been created using lymphocytes from cancer patients. Hagiwara and Sato reported the production of a human monoclonal antibody, CLN-IgG, made by fusing UC 729-6, a 6-thioguanine-resistant human lymphoblastoid B-cell line, with lymphocytes obtained from a patient with squamous-cell carcinoma of the cervix. It has previously been reported that CLN-IgG recognized the antigen expressed in various histological types of human cancers, including malignant gliomas.

In this report, we describe the precise reactivities of CLN-IgG against various human glioma tissues, normal brain tissues, and cultured glioma cells. We define the antigen recognized by CLN-IgG on malignant glioma cells for the purpose of identifying the value of the antibody in the diagnosis and treatment of malignant gliomas.

Materials and Methods

Monoclonal Antibody

The derivation, properties, and purification of the human monoclonal antibody, CLN-immunoglobulin (Ig)G*, have been described in previous reports.

Cells and Tissues

Cells from the human glioma cell lines U-251MG, U-373MG, and KNS-42,† were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and kanamycin (50 μg/ml), and grown in monolayer culture at 37°C in a humidified chamber with 5% CO2 and 95% air atmosphere. Tumor tissues were obtained from glioma patients undergoing surgery at Kobe University Hospital. To obtain primary cultured

* CLN-IgG was kindly supplied by H. Hagiwara, Ph.D.
† Glioma cells obtained from American Type Culture Collection, Rockville, Maryland.
cells, tumor tissues were mechanically cut into fragments of about 0.5 cu mm, incubated for 30 minutes at 37°C in RPMI-1640 medium supplemented with the enzyme Disperse (1000 IU/ml), and passed through a 100-μm stainless steel mesh. The single cells were washed three times with RPMI-1640 medium and suspended in complete medium.

Cell Treatment

The U-251MG cells and primary cultured glioblastoma cells were treated with 1 mM N\(^{3}\),O\(^{2}\)-dibutyryl cyclic adenosine monophosphate (AMP) for 48 hours to induce cell differentiation. Cells at the G\(_2\)/M tumor growth phase were obtained by treatment with serum-free medium for 5 days against confluent cultured cells. Cells at the G\(_2\)/M phase were prepared by mitotic shaking.

Immunoperoxidase Staining

Frozen tissue sections (6 μm) were fixed with cold acetone for 10 minutes, then washed with phosphate-buffered saline (PBS), pH 7.4, for 20 minutes. Sections were preincubated with 0.03% H\(_2\)O\(_2\) in methanol for 20 minutes and washed with PBS for 20 minutes. Following incubation with 5% normal goat serum in PBS for 30 minutes, sections were incubated with 10 μg/ml of CLN-IgG for 60 minutes at 30°C. After another wash with PBS for 30 minutes, sections were incubated with biotinylated goat anti-human IgG for 30 minutes at 30°C. After a further wash with PBS for 30 minutes, sections were incubated with avidin-biotin-peroxidase complex for 30 minutes at 30°C. After yet another wash with PBS for 30 minutes, peroxidase reaction was initiated using 0.06% diaminobenzidine with 0.01% H\(_2\)O\(_2\) in 50 mM Tris-HCl buffer, pH 7.0, for 5 minutes. The sections were briefly counterstained with hematoxylin. Negative control sections were incubated with nonimmunized human IgG in place of CLN-IgG.

Immunofluorescence Staining

Semiconfluent U-251MG cells were grown on cover slips in complete medium, washed with PBS, and fixed with cold 80% ethanol for 20 minutes at 4°C. After being washed with PBS, cells were incubated with 10 μg/ml of CLN-IgG for 30 minutes at 4°C. After another wash with PBS for 30 minutes, sections were incubated with propidium iodine (final concentration of 4 μg/ml). Immunofluorescence and correlated dual-parameter measurements of CLN-IgG versus DNA content were performed with a fluorescence-activated cell sorter.

Flow Cytometry Analysis

Cells were washed three times with PBS, and fixed with 80% ethanol for 30 minutes at 4°C. Staining for CLN-IgG was performed by incubating 1 × 10\(^6\) cells for 60 minutes at room temperature in CLN-IgG, 10μg/ml. After incubation, the cells were washed three times with 0.05% Tween 20/PBS and then incubated for 30 minutes at room temperature in 20 μl of PBS containing a 1:20 dilution of FITC-labeled goat anti-human IgG. Cells incubated with nonimmune human IgG served as a control for background fluorescence.

After being washed three times with 0.05% Tween 20/PBS, the cells were preincubated with 1 mg/ml of ribonuclease for 30 minutes at 30°C then stained with propidium iodine (final concentration of 4 μg/ml). Immunofluorescence and correlated dual-parameter measurements of CLN-IgG versus DNA content were performed with a fluorescence-activated cell sorter.

For analysis of the antigen on the cell membrane, semiconfluent cells were treated with 0.05% ethylene-diaminotetra-acetic acid for 10 minutes at 37°C in order to obtain single cells, then washed with PBS containing 5% fetal bovine serum and 0.05% sodium azide. The cells were incubated for 60 minutes with 5 μg/ml of CLN-IgG at 4°C. After being washed with PBS containing 5% fetal bovine serum and 0.05% sodium azide, the cells were stained with a 1:20 dilution of FITC-labeled goat anti-human IgG for 30 minutes at 4°C. After washing, the signal was measured with the fluorescence-activated cell sorter and stained cells were viewed under the fluorescence microscope.

For analysis of the antigen in cytoplasm, single cells were treated with 0.25% trypsin in PBS for 60 minutes at 37°C following fixation with cold 80% ethanol. Flow cytometry analysis was performed as for the analysis of the antigen on cell membrane. Treatment with 0.25% trypsin for 60 minutes at 37°C as described inhibited the expression of antigen on cell membrane (data not shown).

Immunoblotting

The U-251MG cells (10\(^7\) cells) were solubilized with 500 μl of cell-lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.01% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), and 2 mM phenylmethylsulfonylfluoride. Cyst fluids, sera, and cerebrospinal fluid (CSF) obtained from glioma patients were lyophilized. Samples (1 mg/ml of protein concentration) were dissolved in buffer containing 2.5% SDS, 5% 2-mercaptoethanol and 0.01 M Tris-HCl (pH 8.0), and boiled for 5 minutes at 100°C. The procedure for SDS-polyacrylamide gel electrophoresis used in this study essentially followed the method of Laemmli. The stacking gel contained 5% acrylamide...
Antigen related to cell proliferation

FIG. 1. Immunoperoxidase staining of glioblastoma (A), anaplastic astrocytoma (B), adult normal brain (C), and fetal brain (D) tissues with CLN-IgG. Nuclei were counterstained with hematoxylin. In A, a blood vessel is identified with an arrow. ×150.

and 0.13% N,N'-methylene-bisacrylamide (Bis), and the running gel contained 10% acrylamide and 0.1% Bis. Electrophoresis was performed at room temperature for 4 hours at 20 mA to a 0.4-mm width of gel. The migration front was followed by adding 0.002% bromophenol blue to the electrode buffer. Proteins from the gels were electrophoretically transferred onto nitrocellulose filters at a constant voltage of 50 V for 3 hours as reported by Towbin, et al.26 The nitrocellulose filters were immersed in 3% bovine serum albumin then reacted according to the method described for immunoperoxidase staining.

Antibody-Dependent Cell Cytotoxicity

The U-251MG cells and primary cultured cells from various glioma tissues for use as target cells were mixed with sodium chromate (\(^{51}\)Cr) at 100 \(\mu\)Ci/10\(^6\) cells/ml and incubated for 1 hour at 37°C in a CO\(_2\) incubator. The \(^{51}\)Cr-labeled target cells (1 \(\times\) 10\(^4\)/well) and effector lymphocytes were incubated with or without 50 \(\mu\)g of CLN-IgG for 6 hours at 37°C in a 96-well microplate. Human peripheral lymphocytes collected from healthy donors were used as effector cells. The radioactivity of 0.1 ml of supernatant in each well was collected, and the percentage of lysis was assessed by means of the formula: cytotoxicity (%) = \(((a - c) + (b - c)) \times 100\), in which a is the percentage of release (expressed as counts per minute (cpm)) in the presence of effector cells, b is the percentage of maximum release (in cpm) by 1 N HCl, and c is the percentage of spontaneous cpm with medium alone.

Results

Immunohistochemical Characterization

Table 1 shows the immunohistochemical reactivity of CLN-IgG on various histological types of brain tumor and normal brain tissue. There were 51 brain tumors; seven of the eight glioblastomas (Fig. 1A) and all four anaplastic astrocytomas (Fig. 1B) stained positively. Benign gliomas (oligodendrogliomas, ependymomas, and astrocytomas) showed a low occurrence of CLN-IgG reactivity. The heterogeneous staining by CLN-IgG was typical in tumor cells from glioblastoma specimens (Fig. 1A). Blood vessels in tumor tissue were not stained by CLN-IgG (Fig. 1A). Benign brain tumors (meningiomas, neurinomas, pituitary adenomas, and choroid plexus papillomas) did not stain, but tissue from two of five craniopharyngiomas did. Adult normal brain tissue (Fig. 1C) and fetal brain tissue (Fig. 1D)
FIG. 2. Immunofluorescence staining of fixed U-251MG cells with CLN-IgG. The reactive fluorescein isothiocyanate products with CLN-IgG, especially ruffles, were found on the cell membrane. Nuclei stained with propidium iodide, × 280.

did not show any reactivity with CLN-IgG. The antigen recognized by CLN-IgG was localized in the cell membrane of U-251MG cells (Fig. 2), especially ruffles. The reactivity of CLN-IgG toward U-251MG cells was resistant to treatment with sodium periodate and other modes of deglycosylation (data not shown).

Flow Cytometry

The relationship between the expression of antigen and the cell cycle was examined by flow cytometry. Flow cytometric analysis with the use of CLN-IgG showed strong positive binding to U-251MG and KNS-

<table>
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<tr>
<th>Table 1</th>
<th>Immunohistochemical reactivities of CLN-IgG against various human brain tumors, normal brain, and cultured cells</th>
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<tbody>
<tr>
<td>Histology</td>
<td>No. Positive/ Total Cases</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>7/8</td>
</tr>
<tr>
<td>anaplastic astrocytoma</td>
<td>4/4</td>
</tr>
<tr>
<td>astrocytoma</td>
<td>1/5</td>
</tr>
<tr>
<td>medulloblastoma</td>
<td>1/5</td>
</tr>
<tr>
<td>ependymoma</td>
<td>1/2</td>
</tr>
<tr>
<td>oligodendroglioma</td>
<td>1/3</td>
</tr>
<tr>
<td>metastatic tumor</td>
<td>1/3</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td>3/3</td>
</tr>
<tr>
<td>squamous cell carcinoma</td>
<td>1/2</td>
</tr>
<tr>
<td>craniopharyngioma</td>
<td>2/5</td>
</tr>
<tr>
<td>pituitary adenoma</td>
<td>0/3</td>
</tr>
<tr>
<td>meningioma</td>
<td>0/5</td>
</tr>
<tr>
<td>neurinoma</td>
<td>0/4</td>
</tr>
<tr>
<td>choroid plexus papilloma</td>
<td>0/2</td>
</tr>
<tr>
<td>normal brain (adult)</td>
<td>0/5</td>
</tr>
<tr>
<td>normal brain (fetus)</td>
<td>0/3</td>
</tr>
<tr>
<td>totals</td>
<td>21/59</td>
</tr>
</tbody>
</table>

| U-373MG | positive |
| U-251MG | positive |
| KNS-42 | positive |
| primary cultured cell | |
| glioblastoma | 5/5 |
| anaplastic astrocytoma | 3/3 |
| astrocytoma | 2/3 |

T. Kokunai, N. Tamaki, and S. Matsumoto

42 cells, especially at the G2/M phase compared to that at the G1 phase (Fig. 3). When U-251MG cells were treated with cyclic AMP, they showed reduced expression of the antigen recognized by CLN-IgG, especially the expression of the antigen on cell membrane (Fig. 4). The maximum expression of the antigen on the cell surface was detected in the cells at G2/M phase and synchronously cultured U-251MG cells at the G0/G1 phase showed a markedly decreased expression (Fig. 4). In primary cultured cells from glioblastomas, the expression pattern of the antigen recognized by CLN-IgG was the same as the pattern of U-251MG cells, but more marked expression during the G2/M phase was observed (Fig. 5) than at the G0/G1 phase.

Immunoblotting

Immunoblotting analysis was used to examine the epitope of the antigen in U-251MG cells and the amount of antigen shed into cyst fluids, sera, and CSF of glioma patients (Fig. 6). Cell lysates of U-251MG cells under reduced conditions showed two bands with a molecular weight (MW) of 60 kD and 53 kD. These data suggest that the epitope of the antigen in U-251MG cells recognized by CLN-IgG is the same as that of a previously reported antigen. The antigen recognized by CLN-IgG was detected in the cyst fluid of one glioblastoma, but was a single band with an MW of 60
Antigen related to cell proliferation

![Graph](image)

**FIG. 4.** Binding pattern of CLN-IgG on the antigen of U-251MG cells obtained with flow cytometric analysis. **Upper Left:** Exponentially growing cells. **Lower Left:** Cells treated with cyclic adenosine monophosphate (AMP). **Upper Right:** Cells synchronously accumulated at the Go/G1 phase. **Lower Right:** Cells collected at the G2/M phase by mitotic shaking. FITC = fluorescein isothiocyanate.

kd. Other cyst fluids (six cases), sera (27 cases), and CSF (18 cases) of glioma patients and the supernatant of cultured U-251MG cells showed no reactivity against CLN-IgG.

**Antibody-Dependent Cell Cytotoxicity**

For analysis of the cytotoxic activity of CLN-IgG, we examined the antibody-dependent cell cytotoxicity (ADCC) activity of CLN-IgG against U-251MG and

![Graph](image)

**FIG. 5.** Two-color analysis (left) and binding patterns (right) by flow cytometry of CLN-IgG on the antigen of primary cultured glioblastoma cells. FITC = fluorescein isothiocyanate; AMP = adenosine monophosphate.
primary cultured glioma cells. The ADCC activity of CLN-IgG against U-251MG cells was 10.81% ± 1.24% at an effector:target ratio of 40:1, and was reduced when the cells were treated with cyclic AMP (Fig. 7). Table 2 shows the ADCC activities of CLN-IgG against primary cultured cells from various glioma tissues. The ADCC activity of CLN-IgG against the primary cultured cells of glioblastoma and anaplastic astrocytoma was 36.2% ± 12.85% and 29.4% ± 7.65%, respectively, but against the primary cultured cells of astrocytoma and normal brain CLN-IgG ADCC activity was very low (8.9% ± 3.65% and 4.3% ± 2.81%, respectively).

Discussion

Reactivity of CLN-IgG Antibody

Human monoclonal antibody CLN-IgG reacted with malignant gliomas, especially with glioblastoma and anaplastic astrocytoma. These tumors are the most malignant and invasive among all tumors of neuroectodermal origin. By contrast, CLN-IgG did not react with normal brain tissues. There have been few reports of tumor antigens being detected by human monoclonal antibody. Irie, et al.,11 reported that the L72 antibody (designated anti-OFA-1-2) reacted only with tumor cells of neuroectodermal origin (melanomas, neuroblastomas, and gliomas). In previous studies,1,4,10 CLN-IgG has been reported to react with various human cancers such as cervical carcinoma, gall bladder carcinoma, stomach carcinoma, melanoma, lung carcinoma, and prostate carcinoma. The antigen recognized by CLN-IgG had an MW of 226 kD consisting of α-subunit (MW 60 kD) and β-subunit (MW 53 kD) which were linked by intermolecular disulfide bond. Our immunoblotting results demonstrated that the epitope of the antigen recognized by CLN-IgG in human glioma cells had two molecules of the same sizes as in the previously reported cancer cell antigen.1,10 It is suggested that CLN-IgG recognized the epitope of an antigen that commonly exists in a broad range of malignancies beyond the tumors of neuroectodermal origin.

Therapeutic Application

There is a potential therapeutic application for monoclonal antibodies against cancer cells because cell surface expression of the antigen is thought to be a major factor in the initial reaction of antibody-mediated immunity against cancer cells. Flow cytometric analysis and immunofluorescence staining showed that the antigen was expressed on the cell membrane of U-251MG cells and primary cultured glioblastoma cells. Moreover, flow cytometric analysis revealed that the expression of the cell-surface antigen was higher in the undifferentiated cells than the differentiated cells treated with cyclic AMP. Maximum expression of this antigen on the cell membrane was observed during the G2/M phase of the cell cycle. These data suggest that the function of the antigen recognized by CLN-IgG may be related to cell proliferation.

The shedding of the antigen into body fluid is important for cancer immunotherapy. The antigen recognized by CLN-IgG was shed into the cyst fluid in only one case of glioblastoma and did not shed into the cyst fluid, serum, or CSF of any other case or into the supernatant of U-251MG cells. This shed antigen had a single molecule (MW 60 kD) in contrast to the two molecules detected in the cell lysate of U-251MG cells. This finding suggests the possibility that the shed antigen might be a degraded form of the antigen recognized by CLN-IgG.
Antigen related to cell proliferation

TABLE 2
ADCC activity of CLN-IgG against primary cultured cells from various tissues*

<table>
<thead>
<tr>
<th>Histology of Tissue</th>
<th>No. of Cases</th>
<th>ADCC Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glioblastoma</td>
<td>5</td>
<td>36.2 ± 12.85</td>
</tr>
<tr>
<td>anaplastic astrocytoma</td>
<td>3</td>
<td>29.4 ± 7.65</td>
</tr>
<tr>
<td>astrocytoma</td>
<td>3</td>
<td>8.9 ± 3.65</td>
</tr>
<tr>
<td>normal brain</td>
<td>3</td>
<td>4.3 ± 2.81</td>
</tr>
</tbody>
</table>

* ADCC = antibody-dependent cell cytotoxicity. The effector/target ratio was 40:1. Means are expressed ± standard deviation of the means.

It is important for cancer immunotherapy that CLN-IgG had antibody-dependent cell cytotoxicity against cells and primary cultured cells of glioblastomas and anaplastic astrocytomas. Furthermore, synergistic augmentation was reported in complement-dependent cytotoxicity against cancer cells using CLN-IgG and another human monoclonal antibody. In addition, during the several years since its establishment, the clone has been stable both in CLN-IgG secretion and in specificity against cancer cells.

Radioimaging

Mouse monoclonal antibodies to human cancer cells have been used for immunotherapy and radioimaging. There are some practical problems to using mouse monoclonal antibodies in clinical practice, including the induction of anti-mouse IgG after multiple administrations and the potential adverse reactions against certain normal tissues. The accumulation of anti-mouse IgG in the circulation could neutralize subsequently administered monoclonal antibodies and diminish their effectiveness. Circulating mouse monoclonal antibody-anti-idiotype complexes might lead to immunological suppression, enhancement of tumor growth, and renal dysfunction. For the above reasons, the human-human monoclonal antibody (CLN-IgG) is thought to be of possible use for immunotherapy or radioimaging of malignant gliomas, and for analysis of the humoral immune response against human malignant gliomas such as the anti-idiotype network.

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References


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