Immunohistochemical Study of a Newly Defined Oncofetal Antigen (HGR-Ag) in Tumors of the Central Nervous System

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Abstract

The oncofetal antigen (HGR-Ag) in brain tumors was newly defined using the monoclonal antibody derived from tissue extracts of an anaplastic astrocytoma. The HGR-Ag was purified through sequential chromatography using diethylaminoethyl-Sephadex A-50 and Con-A Sepharose affinity columns. The latter method indicated that HGR-Ag is not a glycoprotein, and Western blot analysis indicated a molecular weight of 80–90 kd. Purified antigen was used to produce antibody. This new antibody, designated H1H2, was shown to be an immunoglobulin G1 by immunodiffusion assay.

Histological/immunohistochemical studies using the H1H2 antibody on paraffin and frozen tissue sections showed HGR-Ag to be intracellular rather than membrane-associated. The immunoreactivity of H1H2 was highest in malignant astrocytomas, glioblastomas, and normal fetal brain tissue. Neurinomas and certain carcinomas of other organs showed lesser, variable reactivity to H1H2. The strength of the antigen-antibody binding was correlated with the degree of malignancy in human gliomas. H1H2 bound to fetal brain tissue and undifferentiated neural tumors, but not to normal adult brain tissue, so HGR-Ag is probably a fetal oncoantigen.

Key words: monoclonal antibody, oncofetal antigen, malignant glioma

Introduction

Several monoclonal antibodies (MoAbs) directed at glioma antigens with cross reactivity to gliomas have been reported.^{8,12,18,21} However, no pure MoAb directed specifically to glioma has yet been established, making clinical applications difficult. However, the use of MoAbs has yielded much information on the biochemistry of malignant tumor cells. Recently, two main clinical approaches to the use of MoAbs have emerged. In immunotherapy, the antibody can damage tumor cells through the immunological response as well as through the direct chemotherapeutic effects of attached anti-tumor agents.^{10,15} In immunohistochemical investigation, the antibody is useful in the diagnosis of tumors that

are difficult to differentiate using morphological characteristics.^{3,5,24}

Here, we report an immunohistochemical study of various brain tumors and malignancies of other organs, normal adult and fetal tissues, and some glioma cell lines using the new MoAb, designated H1H2, which appears to be specifically directed to the oncofetal antigen, HGR-Ag. Additional biochemical characterization of HGR-Ag was also possible.

Materials and Methods

I. Antigen purification

Fresh tissue from a surgically removed anaplastic astrocytoma was homogenized using a Potter-

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Elvehjem homogenizer. After centrifugation at 1000 rpm for 10 minutes, the supernatant containing chiefly soluble proteins was separated by diethyl-aminoethyl (DEAE)-Sephadex A-50 column chromatography into four protein-containing fractions. Protein elution was monitored at 280 nm with a Perkin-Elmer 55B spectrophotometer.¹⁴⁾ The four fractions and supernatants from normal human brain tissue and five malignant gliomas (stored until use at -80°C) were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots (100 μg) of each fraction containing 300 μg of protein were analyzed using 7.5% polyacrylamide gel. Gels were examined to identify bands present in the tumor preparations but not in preparations from normal adult brain tissue. The fraction of the astrocytoma supernatant containing HGR-Ag was further purified using Sephadex G-100 and Con-A Sepharose column chromatography to characterize the antigen.

II. Production of MoAbs

Balb/c male mice were given subcutaneous injections of the purified HGR-Ag (424 μg) suspended in Freund's complete adjuvant (0.4 ml). Seven days later, individuals received an intraperitoneal injection of 1060 μg HGR-Ag. Seven days after this, a second intraperitoneal injection of 212 μg antigen was given. Three days after the third injection, the mice were killed and the splenocytes were harvested. P3X63Ag8.653 mouse myeloma cells (1.0×10^7) were incubated with mouse immune splenocytes (1.0×10^8) in 0.3 ml of 40% polyethylene glycol 1540 for 3 minutes at 37°C .¹³⁾ The resulting cells were seeded into microtiter plates at 1×10^5 cells per well and grown in culture medium (hypoxanthine 1.0×10^{-4} mol/l, aminopterin 1.0×10^{-5} mol/l, thymidine 4.0×10^{-5} mol/l) for selection of hybridomas. Two weeks after the fusion procedure, supernatants from each well were analyzed for antibody by the avidin-biotin complex (ABC) immunoperoxidase technique. Cells were diluted and expanded (3 cycles) to ensure homogeneity of the selected clones of hybridoma cells. Balb/c mice were then injected with the hybridomas for production of ascites fluids. The resulting ascites were purified using a Sephacryl S-300 column equilibrated with phosphate-buffered saline. The purity and specificity of the antibody was determined using the Ouchterlony immunodiffusion assay.

III. Tissue samples

Samples from various brain tumors, malignant tumors of other organs, and normal adult and fetal

brain tissue were fixed in 10% buffered formalin and embedded in paraffin. Brain tumor tissues, stored at -80°C until use, were fixed in periodate-lysine-paraformaldehyde solution and immersed in OCT medium (Miles Co., Eikhart, Ind., U.S.A.).¹⁹⁾ Glioma cell lines, SK-MG-1 and SK-MG-4, were cultured for 4 days in tissue culture chamber slides (4802; Miles Co.), then fixed in 50% methanol and 50% acetone. These slides, and those prepared from frozen sections, were used for immunohistochemical studies.

IV. Immunohistochemical staining

Immunohistochemical staining used the ABC method according to the manufacturer's protocol (Vectastain ABC kit; Vecta Lab. Co., Ltd., Burlingame, Cal., U.S.A.). Briefly, MoAb, diluted at 1:200–1:500, was incubated with the tissue for 1 hour at room temperature. The tissue samples were next incubated with biotinylated secondary antibody, then with performed avidin-biotin horse-radish peroxidase complex. Antigen was localized by incubation in a peroxidase substrate solution.

V. Western blot analysis

The purified proteins separated by SDS-PAGE were transferred electrophoretically to a nitrocellu-

Fig. 1 Electrophoretic analysis using DEAE-Sephadex A-50 column chromatography. Arrows indicate HGR-Ag band. N: normal brain, B: glioma before chromatography, 1-4: gliomas after chromatography.

lose membrane and examined immunohistochemically by the ABC method using H1H2 antibody.⁶⁾

Results

I. Antigen purification

DEAE-Sephadex A-50 column chromatography of the four fractions derived from human astrocytoma identified a band associated with the tumor (HGR-Ag) in the third and fourth fractions (Fig. 1). The fourth fraction was apparently purer and contained more HGR-Ag. Further chromatography of this fraction using the Sephadex G-100 column achieved no adequate separation of the proteins (data not shown). Therefore, the entire protein-containing band was passed through a Con-A Sepharose column, yielding three protein fractions. Only the second fraction contained HGR-Ag by SDS-PAGE analysis (Fig. 2). Addition of 1-0-methyl- α -D-glucopyranoside indicated that the antigen was not a glycoprotein. Western blot analysis showed the molecular weight (MW) of this HGR-Ag to be 85 kd. This fraction was used as the immunogen for production of immune splenocytes in Balb/c mice. Antibody H1H2 was obtained following fusion of immune splenocytes and mouse myeloma P3X63Ag8.653 cells as described previously.

Fig. 2 Final fraction after Con-A Sepharose column chromatography. Arrow indicates HGR-Ag band.

Fig. 3 upper: Astrocytoma grade II. Immunoreactivity in the cytoplasm of tumor cells is much more intensive. Note that the nuclei of tumor cells do not stain. ABC stain, $\times 400$. lower: Glioblastoma. The cytoplasm of the tumor cells is strongly stained. ABC stain, $\times 400$.

II. Characterization of H1H2

Ouchterlony immunodiffusion assay showed the ascites-derived MoAb H1H2 to be immunoglobulin G1. Immunohistochemical studies showed that H1H2 reacted specifically with cytoplasmic components or interstitial tissues in tumors (Fig. 3). In paraffin sections of brain tumors, the immunoreactivities of astrocytoma grades I and II, astrocytoma grades III and IV, and glioblastoma were 45, 85, and 90% positive staining, respectively (Table 1). The data also indicated that the reactivity of H1H2 to gliomas was positively correlated with malignancy. Thus, although neurinomas demonstrated some reactivity, no medulloblastoma or ependymoma tested exhibited a positive reaction. H1H2 binding in frozen brain tumor sections generally corresponded to that in paraffin sections. Among malignant tumors in other organs, some hepatomas and small cell carcinomas showed positive antibody-mediated staining, but most tumors did not (Table 2). Some carcinomas showed lesser activity. Glioma cell lines, SK-MG-1 and SK-MG-4, demonstrated positive reac-

Table 1 Immunoreactivity of H1H2 in paraffin and frozen sections of human brain tumors

Histology	No. of positive/ total cells	Positive percentage
Paraffin section:		
Astrocytoma		
grades I and II	9/20	45
grades III and IV	17/20	85
Glioblastoma	9/10	90
Oligodendroglioma	3/10	30
Ependymoma	0/10	0
Medulloblastoma	0/10	0
Neurinoma	2/10	20
Pituitary adenoma	0/8	0
Meningioma	0/10	0
Hemangioblastoma	0/10	0
Frozen section:		
Astrocytoma		
grades I and II	5/10	50
grades III and IV	9/10	90
Glioblastoma	7/7	100
Ependymoma	0/5	0
Neurinoma	0/5	0
Meningioma	0/5	0

Table 2 Immunoreactivity of H1H2 in paraffin sections of carcinomas of other organs

Organ	No. of positive/total cells
Hepatoma	4/8
Thyroid carcinoma	0/7
Esophagus	
adenocarcinoma	0/3
squamous cell carcinoma	0/5
Stomach	
adenocarcinoma	0/7
Colon	
adenocarcinoma	0/6
Melanoma	0/5
Renal cell carcinoma	0/5
Bladder carcinoma	0/5
Malignant lymphoma	3/10
Ovary carcinoma	0/3
Lung	
squamous cell carcinoma	0/5
adenocarcinoma	0/5
small cell carcinoma	3/5
Uterus	
adenocarcinoma	0/10

tivities, although the patterns of staining were reticular or granular, in comparison to the more homogeneous patterns observed in paraffin or frozen

Fig. 4 upper: Glioma cell line, SK-MG-1. The binding of H1H2 to tumor cells is marked, showing a granular or reticular pattern. ABC stain, $\times 1000$. lower: Glioma cell line, SK-MG-4. The tumor cells are strongly stained like SK-MG-1. ABC stain, $\times 1000$.

sections (Fig. 4). With the exception of fetal brain tissue, normal human adult and fetal tissues were non-reactive (Table 3).

III. SDS-PAGE analysis of human gliomas

Four of the five gliomas examined contained HGR-Ag, shown by the band at the appropriate MW (Fig. 5). These four gliomas showed positive H1H2-mediated immunohistochemical staining, while the fifth glioma was negative. Therefore, the immunohistochemical analysis was corroborated by the SDS-PAGE analysis, showing the accuracy of the ABC technique.

Discussion

The use of MoAbs to study the central nervous system (CNS) has yielded much information on CNS differentiation, differential diagnosis and microbiology of CNS pathology, particularly of CNS tumors. Antigens expressed by CNS tumors, especially gliomas, are usually in one of four categories:

Table 3 Immunoreactivity of H1H2 in paraffin sections of normal human tissues

Organ	No. of positive/total cells	
	Adult	Fetal
Brain	0/5	4/5
Kidney	0/5	0/5
Spleen	0/5	0/5
Thymus	0/5	0/5
Colon	0/5	0/5
Esophagus	0/5	0/5
Bladder	0/5	0/5
Liver	0/5	0/5
Lung	0/5	0/5
Muscle	0/5	0/5
Skin	0/5	0/5

Fig. 5 Electrophoretic analysis of various gliomas. Arrow indicates HGR-Ag band. 1-5: malignant gliomas, 6: edematous brain tissue around glioma 5, N₁: normal frontal lobe, N₂: normal basal ganglia.

1) normal CNS-associated antigens,^{22,23)} 2) shared neural/lymphoid antigens, 3) neuroectodermal-oncofetal antigens, or 4) putative tumor-specific antigens.^{9,25)} At present, the most widely used tumor markers for immunohistochemical studies are MoAbs or polyclonal antisera directed against glial fibrillary acidic protein (GFAP)^{3,17)} and neuron-specific enolase,^{4,20)} all considered normal CNS-asso-

ciated antigens and components of cell cytoplasm. Most antigens in the other categories are glycoproteins, glycolipids, or carbohydrates localized on or within the cell surface membranes.

Immunohistochemical investigation has shown H1H2 binds to a cytoplasmic moiety or moieties, in contrast to the antibodies to GFAP and S-100 protein, which have shown cross reactivity to non-cytoplasmic entities.²⁾ The high affinity of H1H2 for malignant gliomas, including anaplastic astrocytomas and glioblastomas, and the non-reactivity in normal adult brain, suggest that the target antigen, presumably HGR-Ag, is also specific to more undifferentiated tissues. However, H1H2 does not cause immunoreactivity in human ependymoma or medulloblastoma, the latter being one of the most undifferentiated neural malignancies. Neurinomas showed some low-level binding, but all other neural tumors tested were negative. Significantly, H1H2 shows no apparent binding to normal adult brain tissues, but bound strongly in 12-18-week fetal brains, indicating that HGR-Ag is a fetal antigen or oncoantigen. Glial tumors in the CNS infiltrate the surrounding normal tissue, so such tumors include transformed cells and normal cells of various types. Unless the antigen or antigens are specific to the tumor cells only, some non-specificity of antibodies can be expected. The histogenesis of the cell expressing the antigen would also determine the level of immunoreactivity.

Other CNS-specific antibodies available for immunohistochemical studies include UJ127:11 and UJ13A, both established from as yet unspecified antigens isolated from fetal brain.¹¹⁾ UJ13A binds in neural tumors derived from neuroectodermal cells, for example gliomas, ependymomas, meningiomas, and neurinomas. In contrast, UJ127:11 binds to tumors such as medulloblastomas and neuroblastomas. However, both antibodies react with a broad range of normal tissues as well, including adult and fetal brain, adrenal gland, and pancreas.^{1,11)}

Cell lines have also produced some relatively tissue-specific antibodies. Carrel *et al.*^{8,9)} isolated antibodies, designated Mel-4, Mel-14, and Me3-TB7, derived from melanoma cultures and binding to gliomas, neuroblastomas, and melanomas. Liao *et al.*¹⁶⁾ produced MoAbs 7.51 and 7.60 from melanoma cultures, which react to the same types of brain tumors and to fetal brain. The antigens recognized by these MoAbs are evidently common to both glial and neuronal tumors and suggest a neuroectodermal connection in melanomas. MoAbs such as CG12, AO10, and G13-06, produced by immunizing animals with glioma cell lines, recognize an-

tigens in gliomas, melanomas, neuroblastomas, and fetal brain-like melanoma-derived MoAbs. However, AO10 and another glioma-derived antibody, AJ8, also show affinity for astrocytomas and epithelial cancers.⁷⁾ These MoAbs differ in that AJ8 binds more to the differentiated and/or GFAP-positive gliomas while AO10 has greater affinity for undifferentiated, GFAP-negative gliomas. H1H2, in this respect, resembles AO10, but H1H2 does not react to tissues of neuroectodermal origin.

This study has further defined the oncofetal antigen, HGR-Ag, by antigen purification from a human astrocytoma, and demonstrated its existence in several tissues by immunohistochemical methods supported by column chromatography and gel electrophoresis. A new MoAb to HGR-Ag, H1H2, has a high affinity for malignant gliomas and shows a positive correlation between immunoreactivity and tumor malignancy. The affinity of H1H2 for normal fetal brain and undifferentiated CNS tumors also suggests that HGR-Ag is a fetal oncoantigen. H1H2 is another MoAb to human neural tumors, which will help in the differentiation, diagnosis, and biochemical investigation of CNS tumors.

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