Lectin Cytochemistry Combined with Silver Colloid Staining of Argyrophilic Nucleolar Organizer Regions in Human Gliomas

Shuji NIKAWA, Akira HARA, Takashi ANDO, Noboru SAKAI, Hiromu YAMADA and Kuniyasu SHIMOKAWA*

Departments of Neurosurgery and *Pathology, Gifu University School of Medicine, Gifu

Abstract

Investigation of lectin cytochemical staining of inflammatory cells in human gliomas showed that Allomyrina dichotoma (Allo-A) cytochemistry can reliably distinguish inflammatory from neoplastic cells. Allo-A cytochemistry combined with silver colloid staining of argyrophilic nucleolar organizer regions (Ag-NORs) was performed in human gliomas. Inflammatory cells possessed usually one but sometimes two Ag-NORs and macrophages often possessed several Ag-NORs. The mean Ag-NOR number per nucleus of inflammatory cells ranged from 1.81 to 2.34, and that of neoplastic cells ranged from 2.57 to 3.53 and from 2.84 to 4.46 in low- and high-grade gliomas, respectively. The mean Ag-NOR number per nucleus of inflammatory cells was significantly smaller than that of neoplastic cells (p < 0.001). Combined Allo-A cytochemical and silver colloid Ag-NOR staining can provide a reliable Ag-NOR number in human gliomas by distinguishing inflammatory cells.

Key words: lectin, inflammatory cells, human glioma, nucleolar organizer regions

Introduction

The presence of inflammatory cells in human brain tumors is well known.1,7,21,28,32,33) Nearly all tumors have a high content of macrophages and a relatively low content of lymphocytes.21,28) Inflammatory cells are considered important in the host immune reaction to tumors.1,6,20,21,24,25,32,33) Morphological discrimination of these inflammatory cells in tumors is of great diagnostic importance. However, inflammatory cells, especially macrophages, are difficult to distinguish from neoplastic cells because macrophages exhibit considerable morphological heterogeneity.21,26,32,33)

Argyrophilic nucleolar organizer regions (Ag-NORs), appearing as black dots in cell nuclei after silver colloid staining, reflect cellular proliferative potential or malignancy.2,3,14,15,17,23) Investigations of the correlation between glioma grade and Ag-NORs have given conflicting results.14,15,17,19) Silver colloid Ag-NOR staining cannot distinguish inflammatory from neoplastic cells. Presumably, one misleading factor is that many inflammatory cells are present even in intact tumor tissue.

Immunoperoxidase staining for α1-antitrypsin, α1-antichymotrypsin, and lysozyme can identify macrophages, but false-negative results may occur.11,16,24,26,27) Cell surface markers have two major types: surface antigens detectable by antibodies, and surface carbohydrates detectable by lectins.29) At present, several antibodies for both macrophage and lymphocyte are available.7,11,28) Cellular carbohydrate moieties act as receptors, antigens, or structural framework components.4,8-10,34) Lectins, the specific carbohydrate-binding proteins or glycoproteins,4) are reliable markers for macrophages and lymphocytes.16,24-27,29,31)

We therefore investigated to see whether a combined lectin cytochemical and silver colloid Ag-NOR staining can obtain a reliable Ag-NOR number in human gliomas by distinguishing inflammatory cells.

Materials and Methods

I. Tumors

All brain tumors in this study were removed
surgically at the Gifu University Hospital, and included glioblastomas (n = 15), anaplastic astrocytomas (9), benign astrocytomas (7), medulloblastomas (4), ependymomas (3), and oligodendrogliomas (2). The tumor tissues were fixed in 10% formalin, embedded in paraffin, and cut into 3-μm sections.

II. Lectin staining and immunostaining for inflammatory cells

Table 1 summarizes the lectins studied: **Allyomyrina dichotoma** (Allo-A) (Cosmo Bio, Tokyo), *Ricinus communis* (RCA-1), *Canavalia ensiformis* (concanavalin A: Con A), *Arachis hypogaea* (peanut: PNA), and *Phytolacca americana* (pokeweed: PWA) (EY Lab., San Mateo, Cal., U.S.A.). Lectin staining used the avidin-biotin peroxidase complex (ABC) method with some modifications. Paraffin sections were dewaxed in xylene and hydrated by a graded ethanol series and phosphate buffered saline (PBS). The sections were incubated in methanolic 0.5% H2O2 to block endogenous peroxidase for 30 minutes. After incubation with 1% bovine serum albumin (BSA) for 30 minutes, sections were reacted with biotin-labeled lectins at a final concentration of 10-12 pg/ml (in PBS containing 0.1% BSA) at room temperature for 45 minutes. After washing five times with PBS, sections were treated with ABC (Sigma Chemical Co., St. Louis, Mo., U.S.A.) containing 0.1% BSA for 30 minutes at room temperature. After rinsing with PBS, 3,3’-diaminobenzidine tetrahydrochloride (DAB) was used as substrate for the peroxidase, and the sections were counterstained lightly with hematoxylin. The specificity was tested by either omitting the labeled lectin or blocking the lectin binding with an appropriate concentration of complementary sugar.

Immunostaining for inflammatory cells used sections treated with hydrogen peroxide in methanol, incubated sequentially with normal horse serum, mouse anti-human macrophage (M814; Dako, Glostrup, Denmark) or lymphocyte sera (M 742 for T-cell and M 755 for B-cell; Dako), and then incubated with biotinylated horse antibody against mouse immunoglobulins (Vector Lab., Burlingame, Cal., U.S.A.) and ABC solution. DAB was used as substrate for the peroxidase. Additionally, immunostaining for glial fibrillary acidic protein (GFAP) (anti-GFAP serum; Dako) was performed to clarify the distribution of the glial cells.

III. Combined Allo-A cytochemical and silver colloidal Ag-NOR staining

Fourteen human high- and low-grade gliomas were used for combined Allo-A cytochemical and silver colloidal Ag-NOR staining. After Allo-A staining, the sections were immersed in deionized water. The silver colloid solution for Ag-NOR staining was prepared by dissolving 2% gelatin in 1% aqueous formic acid and mixing with 2 vol. of 50% aqueous silver nitrate. The sections were coated with Ag-NOR stain and left for 1 hour under safelight conditions at room temperature.

The numbers of Ag-NORs in at least 200 cells on each section were counted under ×1000 magnification. Data were expressed as means ± SD, and the Welch’s or Student’s t-test used for statistical analysis. A p value of less than 0.05 was taken as a significant difference.

### Results

I. Staining of inflammatory and neoplastic cells

All five lectins stained inflammatory cells and macrophages. Lymphocytes were stained by Allo-A (Fig. 1), RCA-1, and Con A, but not by PNA and PWA. Neoplastic cells were stained by RCA-1, Con A, and PNA, but not by Allo-A and PWA. RCA-1 and PNA stained inflammatory cells more intensely than neoplastic cells, and Con A stained inflammatory cells and neoplastic cells similarly. The comparison with immunostaining for inflammatory cells showed that Allo-A stained most or all inflammatory cells (Figs. 1 and 2).

Inflammatory cells were scattered even in intact tumor tissue, especially in malignant gliomas and astrocytomas. Lectin binding and the morphological appearance of macrophages were unrelated, as lectins stained macrophages of varying size and shape with altered cytoplasm. In addition, lectins stained vascular endothelia, and therefore the distribution of both inflammatory cells and vascular endothelia could be demonstrated.

---

Table 1: Summary of lectins used

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Nominal carbohydrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allo-A</td>
<td>NeuAcα2 → 3Galβ1 → 4GlcNAc</td>
</tr>
<tr>
<td></td>
<td>(GlcNAcβ1 → 4GlcNAc)₃</td>
</tr>
<tr>
<td>RCA-1</td>
<td>βGal &gt; αGal &gt; GalNAc</td>
</tr>
<tr>
<td>Con A</td>
<td>αMan &gt; αGlc &gt; GlcNAc</td>
</tr>
<tr>
<td>PNA</td>
<td>Galβ1 → 3GlcNAc &gt; α/βGal</td>
</tr>
</tbody>
</table>


---

Neurol Med Chir (Tokyo) 32, August, 1992
II. Enumeration of Ag-NORs in the Allo-A-positive or negative cells

Allo-A stained inflammatory cells but not neoplastic cells, making distinction easy. The positive Ag-NORs were present as clear black dots in the cell nuclei. Lymphocytes generally possessed one Ag-NOR while macrophages often possessed several Ag-NORs (Fig. 3). The mean Ag-NOR number per nucleus of Allo-A-positive inflammatory cells ranged from 1.81 to 2.34, and that of Allo-A-negative neoplastic cells ranged from 2.57 to 3.53 and from 2.84 to 4.46 in low- and high-grade gliomas, respectively (Table 2, Fig. 4). The mean Ag-NOR number of inflammatory cells was significantly smaller than that of neoplastic cells (p < 0.001).

Discussion

This study showed that macrophages were stained by the five lectins used, and lymphocytes were stained...
Neoplastic cells in gliomas were stained by RCA-1, Con A, and PNA, but not by Allo-A and PWA. Therefore, Allo-A cytochemistry can reliably distinguish inflammatory cells, both lymphocytes and macrophages, from neoplastic cells.

The analysis of tumor proliferation or malignancy in tissue has been widely studied. Immunohistochemical detection of Ki-67 antigen and incorporation of bromodeoxyuridine are methods for assessing the proliferative potential. Recently, Ag-NORs in the cell nuclei have been reported to reflect cellular proliferation and malignancy, but the correlation between glioma grade and Ag-NORs is uncertain.

One-step silver colloid staining cannot distinguish inflammatory from neoplastic cells. Evaluation of tumor proliferative potential or malignancy should include only neoplastic cells. This study shows that without discrimination of inflammatory cells, the mean Ag-NOR number may be smaller than actual, especially in high-grade gliomas, leading to false results. This may also be true for other methods analyzing cellular kinetics in brain tumors.

This study showed that lectins, especially Allo-A, can identify inflammatory cells in human gliomas. The combined technique, Allo-A cytochemistry and silver colloid Ag-NOR staining, can obtain a reliable Ag-NOR number in human gliomas by distinguishing inflammatory cells.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Allo-A-positive cells</th>
<th>Allo-A-negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.06 ± 0.98</td>
<td>3.72 ± 1.42</td>
</tr>
<tr>
<td>2</td>
<td>2.10 ± 1.05</td>
<td>4.02 ± 1.49</td>
</tr>
<tr>
<td>3</td>
<td>1.94 ± 0.87</td>
<td>3.34 ± 1.10</td>
</tr>
<tr>
<td>4</td>
<td>1.88 ± 0.78</td>
<td>4.46 ± 1.67</td>
</tr>
<tr>
<td>5</td>
<td>2.18 ± 0.94</td>
<td>3.64 ± 1.39</td>
</tr>
<tr>
<td>6</td>
<td>2.34 ± 0.98</td>
<td>4.29 ± 1.75</td>
</tr>
<tr>
<td>7</td>
<td>2.10 ± 0.89</td>
<td>3.42 ± 1.26</td>
</tr>
<tr>
<td>8</td>
<td>1.98 ± 0.90</td>
<td>3.89 ± 1.80</td>
</tr>
<tr>
<td>9</td>
<td>2.10 ± 0.99</td>
<td>2.84 ± 1.39</td>
</tr>
<tr>
<td>(mean)</td>
<td>(2.08)³</td>
<td>(3.74)³</td>
</tr>
</tbody>
</table>

⁴Cases 1–9: anaplastic astrocytoma and glioblastoma, Cases 10–14: benign astrocytoma. ⁵Significantly different in each case (p < 0.001). ⁶Not significantly different. ⁷Significantly different (p = 0.005).

by Allo-A, RCA-1, and Con A. Neoplastic cells in gliomas were stained by RCA-1, Con A, and PNA, but not by Allo-A and PWA. Therefore, Allo-A cytochemistry can reliably distinguish inflammatory cells, both lymphocytes and macrophages, from neoplastic cells.

The analysis of tumor proliferation or malignancy in tissue has been widely studied. Immunohistochemical detection of Ki-67 antigen and incorporation of bromodeoxyuridine are methods for assessing the proliferative potential. Recently, Ag-NORs in the cell nuclei have been reported to reflect cellular proliferation and malignancy, but the correlation between glioma grade and Ag-NORs is uncertain.

One-step silver colloid staining cannot distinguish inflammatory from neoplastic cells. Evaluation of tumor proliferative potential or malignancy should include only neoplastic cells.

This study shows that without discrimination of inflammatory cells, the mean Ag-NOR number may be smaller than actual, especially in high-grade gliomas, leading to false results. This may also be true for other methods analyzing cellular kinetics in brain tumors.

This study showed that lectins, especially Allo-A, can identify inflammatory cells in human gliomas. The combined technique, Allo-A cytochemistry and silver colloid Ag-NOR staining, can obtain a reliable Ag-NOR number in human gliomas by distinguishing inflammatory cells.

Table 2 Number of Ag-NORs per nucleus of Allo-A-positive and negative cells within the same samples

![Image of Table 2]

Fig. 4 Allo-A staining (left), and combined Allo-A cytochemical and silver colloid Ag-NOR staining (center, right) in a glioblastoma. Left: Many inflammatory cells are present. Arrowheads indicate endothelial proliferation. ×150. Center: Inflammatory cells stained by Allo-A often possess several Ag-NORs. ×300. Right: Neoplastic cells have an equal or greater number of Ag-NORs but are not stained by Allo-A. Three macrophages (arrowheads) with several Ag-NORs are seen. ×360.
References


32) Wood GW, Gollahon KA: Detection and quantita-


Address reprint requests to: S. Niikawa, M.D., Department of Neurosurgery, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan.