Ganglioside GD3 Immunocytochemistry does not Visualize Microglia but Astroglia

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Ganglioside GD3, which has been used as “a marker for oligodendrocyte progenitor cells” *in vitro*, is present in a few cells under the ependymal lining of the ventricle in adult rat brains. However, recent studies using a monoclonal antibody against GD3 (R24) suggested that GD3-positive cells in adult rat brains are microglia, thus making the cellular localization of GD3 controversial. In this immunohistochemical study, we compared images of immunostained sections of adult rat brains with a monoclonal antibody against GD3 (DSG-1) with those immunostained with

microglia-specific (antibody against thymosin β 4), astroglia-specific (antibodies against GFAP or S-100 protein) or with oligodendroglia-specific (antibodies against GalC or MBP) antibodies for the purpose of re-examining the identity of GD3-positive cells in adult rat brains. We observed co-localization of GD3 immunoreactivity with that of GFAP or S-100 protein, but not with that of thymosin β 4, MBP or GalC. These results indicate that the GD3-positive cells in adult rat brains are astroglia, not microglia or oligodendroglia.

Key words: Adult rat brain, Astroglia, Ganglioside GD3, Immunohistochemistry, Microglia

I. Introduction

Ganglioside GD3 has been used as “a marker of oligodendrocyte progenitor cells” *in vivo* and *in vitro* studies. GD3 was reported to be expressed not only in oligodendroglial progenitor cells but also in astroglial progenitor cells, and to be associated with GFAP in primary cultured cells from neonatal and postnatal rat brains [1, 4, 6, 15]. *In situ* immunohistochemical studies using developing rat brains detected GD3 in immature cells of neuroectodermal origin, including glial progenitor cells [5, 6, 11–13]. It has been observed that the expression level of GD3 decreases with age [5], becoming localized in the subventricular zone in young rat brains and appearing in a few cells just under the ependymal layer of the ventricle in adult rat brains [5]. Using a monoclonal antibody against GD3 (R24) another group reported that in both young and adult rats, GD3-immunoreactivity was detected in microglia and stated that GD3-positive cells were of microglial, and not oligodendroglial lineage [17, 18]. The

identity of GD3-positive cells is thus still controversial.

In this study we attempted to verify whether microglia in adult rat brains are really GD3-positive or not, using double immunofluorescence staining methods with a different monoclonal antibody (DSG-1) against GD3 and antibody against thymosin β 4 (Tb4), which are believed to be specific for microglia [14]. Antibody against astroglia-specific markers, GFAP or S-100 protein, or antibody against oligodendroglia-specific markers, galactocerebroside (GalC) or myelin binding protein (MBP) was also prepared for double immunostaining with DSG-1. Our results confirmed that in adult rat brains, GD3 is co-localized with GFAP in astroglial processes, but is not found in Tb4, GalC or MBP-positive cells, indicating that GD3-positive cells are astroglia, and not microglia or oligodendroglia.

II. Materials and Methods

Animals

Under anesthesia with ether, 10 week-old Sprague-Dawley rats (Jana Clea Laboratories, Osaka) were perfused transcardially with 0.1 M phosphate buffer saline (PBS, pH 7.4) for 5 min and then with 4% paraformaldehyde.

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hyde in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min. Brains were removed and further immersed in the same fixative for 45 min at 4°C. After subsequent immersion in 15% sucrose/PB for 1 day, the brains were frozen at -80°C and stored. Samples containing cerebral cortex, hippocampus, cerebellum and brain stem were examined.

Antibodies

For the primary antibodies against ganglioside GD3, two types of monoclonal antibodies, DSG-1 [16] and GMR 19 (Seikagaku Corp., Tokyo, Japan), were prepared. A rabbit anti-Tb4 polyclonal antibody was generated using a peptide containing amino acids 16-43 of rat Tb4 [19] as the immunogen, and used for identification of microglia. For detection of astroglia, anti-bovine S-100 protein polyclonal antibody (DAKO, Glostrup, Denmark) and anti-GFAP monoclonal antibody (Progen Biotechnik GmbH, Heidelberg, Germany) were used. Anti-GalC and anti-MBP monoclonal antibodies (Behringer Mannheim, Mannheim, Germany) were used to detect oligodendroglia.

The following secondary antibodies and fluorescent probes were used: biotinylated goat anti-mouse IgM (u chain specific, Zymed Lab. Inc., San Francisco, USA), biotinylated goat anti-rabbit IgG (Vector, Burlingame, USA), Alexa[™] 488 goat anti-mouse IgG (Molecular Probes, Oregon, USA), Alexa[™] 488 goat anti-mouse IgG (Molecular Probes, Oregon, USA) and avidin-conjugated Alexa[™] 594 (Molecular Probes, Oregon, USA).

Immunohistochemistry

For conventional microscopy, the fixed rat brains were cryosectioned at 20 µm in thickness and permeated

with 0.3% triton X-100 in PBS for 24 hr. These sections were incubated in DSG-1 (1 : 500), GMR 19 (1 : 500) or anti-Tb4 (1 : 15,000) antibody overnight at 4°C and the immunoproteins were detected by the ABC method employing the Biotin/Avidin System reagents (Vector, Burlingame, USA). Immunolabeled sections were examined by conventional microscopy.

For double immunofluorescence staining, the sections were treated with 0.3% triton X100 in PBS for 24 hr and incubated with DSG-1 (mouse IgM, 1 : 500) overnight at 4°C and washed with PBS, followed by incubation with biotinylated anti-mouse IgM antibody for 1 hr at room temperature. After intensive washing with PBS, they were incubated with another primary antibody, anti-S-100 protein antibody (rabbit IgG, 1 : 15,000), anti-GFAP (mouse IgG, 1 : 2), anti-Tb4 (rabbit IgG, 1 : 10,000), anti-GalC (mouse IgG, 1 : 100) or anti-MBP (mouse IgG, 1 : 500) antibody diluted in PBS, overnight at 4°C. After washing, sections were incubated in a mixture of the following fluorescent probes, Alexa[™] 488 goat anti-rabbit IgG and avidin-conjugated Alexa[™] 594, or Alexa[™] 488 goat anti-mouse IgG and avidin-conjugated Alexa[™] 594 for 1 hr at room temperature. To perform a detailed analysis of double-immunostained sections, Z-series of optical slices were collected on a confocal laser scanning microscope, Fluoview (Olympus, Tokyo, Japan).

III. Results

Anti-Tb4 polyclonal antibody specifically immunostains microglia

Immunostaining using newly prepared polyclonal antibody against Tb4 visualized a subclass of glial cells,

Fig. 1. Immunohistochemistry of Tb4 in rat brain stem, visualizing profiles of ramified microglia. (ABC method).

which had small nuclei, elongated cell bodies and thin, twisted processes with tiny pointed spines (Fig. 1). These features are identical with those of ramified microglia visualized by classical silver impregnation method [3]. The antibody did not stain GFAP-positive astroglia (Fig. 2). Thus, the anti-rat Tb4 antibody, newly generated in our laboratory, is considered to label microglia specifically.

GD3-positive cells are present in the cortex

On the sections immunostained with GSD-1, the clearest figures of GD3-positive cells were obtained in the cerebral cortex, especially in the area near the pia matter (Fig. 3). These cells had relatively large amounts of perinuclear cytoplasm and a few long, slender processes which showed branching; but, spines were not evident. Numerous small fragments of GD3-positive processes were observed in the same area. Classification of these cells on the basis of their stained profile on this immunohisto-

Fig. 2. Fluorescence images of the cerebral cortex double immuno-fluorescence stained with anti-Tb4 and anti-GFAP antibodies. Green images show Tb4 (a, c) and red images show GFAP (b, c). GFAP- and Tb4-positive cells are distinct from each other.

Fig. 3. Immunohistochemistry with DSG-1 applied on rat cerebral cortex. Long GD3-positive processes are observed. Fragments of GD3-positive processes are also found on the neuropile. (ABC method).

Fig. 4

chemistry was difficult. Similar results were obtained with GMR 19.

GD3-positive processes belong to astroglia

To determine to which class the GD3-positive cells belong, double immuno-fluorescence stainings with DSG-1 and antibody against Tb4, GFAP, S-100 protein, GalC or MBP were carried out. Contrary to our expectations, GD3-positive cells were clearly distinct from Tb4-positive microglia (Fig. 4-1a, b, c) or from GalC or MBP-positive oligodendroglia (data not shown). Instead, we observed that GD3-positive cells were also GFAP-positive (Fig. 4-2a, b, c and 4-3a, b, c). The intracellular distribution of GD3 in GFAP-positive cells, however, differs among astroglia located in different regions of the brain. For example, in the astroglia of the cerebral cortex most GFAP-positive processes are also positive for GD3, whereas in the stratum radiatum of the hippocampus, GD3 immunoreactivity is not detected in some GFAP-positive processes (Fig. 4-3a, b, c). Most S-100 protein-positive cells are also GD3-positive (Fig. 4-4a, b, c), where GD3 tended to be distributed in the perinuclear cytoplasm and the proximal part of the processes.

In addition to astroglia, pyramidal neurons in the hippocampus (Fig. 4-3a, b, c), a few nerve cells in the cerebral cortex and ependymal cells exhibit GD3 immunoreactivity (data not shown). Like microglia in the cerebral cortex and hippocampus, microglia in the cerebellum and facial nucleus did not express GD3 (data not shown).

IV. Discussion

The present immunohistochemical study using DSG-1, a monoclonal antibody against GD3, disclosed that GD3 was co-localized with GFAP in the processes and perinuclear cytoplasm of astroglia. GD3 immunoreactivity was not observed in the microglia visualized by Tb4 immunohistochemistry. This result indicates strongly that microglia do not express GD3, contrary to previous reports [17, 18], and also revealed the presence of GD3 in astroglia. Although GD3/GFAP-double positive cells have been observed in primary cultured cells from neonatal and postnatal rat brain [1, 4, 6, 15], this is the first report showing GD3/GFAP-double positive cells in normal adult rat brains. Since co-localization of GD3 and GFAP in a subset of astroglia has been reported in mice *in vivo* [2], GD3 might be generally expressed in astroglia of rodent

brains. The distribution and quantity of GD3 were varied in astroglia which located in different parts of brains. It has been suggested that GD3 inhibits polymerization of glial filaments [7]. If it is the case, differences in GD3 expression among GFAP positive astroglia might reflect the different turnover pattern of GFAP among the astroglia located in different parts of the brain.

In previous immunohistochemical studies, where R24 was used as the primary antibody against GD3, GD3 immunoreactivity was detected in the microglia [17, 18]. The discrepancy between our present results and those studies might be due to the following differences in the nature of antibodies used in the studies: ① the antibody DSG-1, which we used, reacts only with GD3 [16], whereas R24 has been shown to react with other subclasses of ganglioside, GT1a and GQ1b, in addition to GD3 [16], and ② DSG-1 is a mouse IgM [16], whereas R24 is a mouse IgG.

Using DSG-1 as the primary antibody, Kawai *et al.* reported that the reactive astroglia were positive for GD3, but that the astroglia in the normal adult rat brains were GD3-negative [8–10]. Our present GD3 immunohistochemistry could visualize astroglial processes present in normal adult rat brains. The staining conditions, such as TritonX-100 treatment, employed in this study might be better suited to staining GD3 than the procedures previously reported [8–10].

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VI. References

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Fig. 4. The immunofluorescence image of double staining applied on the cerebral cortex (1, 2, 4) and hippocampus (3). Right column: The merged images, double-stained by DSG-1 and anti-Tb4 antibody (1c), DSG-1 and anti-GFAP antibody (2c, 3c), or DSG-1 and anti S-100 protein antibody (4c). Red image shows GD3. Green image in (1) represents Tb4, in (2) and (3) GFAP and in (4) S-100 protein. Yellow images show double positive (red and green) areas. Left and middle columns show each component separately; left column 1a–4a: GD3, middle column 1b: Tb4, 2b and 3b: GFAP, 4b: S-100. Tb4 positive cells are not GD3-positive (1a–c). GD3 is observed in the processes and perinuclear cytoplasm of GFAP-positive cells (2a–c, 3a–c) or S-100 protein-positive cells (4a–c). In the hippocampal astroglia, GD3 is not observed in some GFAP-positive processes of astroglia (3b, c thick arrow). Pyramidal neurons are also GD3-positive (3a, c thin arrow).

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