LOCALIZATION OF METALLOTHIONEIN IN THE BRAIN OF MACACA FASCICULARIS

KEIJI SUZUKI¹, KATSUYUKI NAKAJIMA², UMEKO KAWAHARADA¹,
FUMIKO HARA¹, KATSUYOSHI UEHARA¹, NORIKO OTAKI³,
MASAMI KIMURA⁴ AND YUJI TAMURA⁵

Department of Pathology¹, College of Medical Care and Technology, Gunma University, Showamachi 3-39-15, Maebashi 371, Japan Immunoresearch Laboratories², Sakaicho 17, Takasaki 370, National Institute of Industrial Health³, Tama-ku Nagao 6-12, Kawasaki, 214, Central Institute for Experimental Animals⁴, Nogawa 1430, Miyamaeku, Kawasaki 213, and 1st Department of Pathology⁵, School of Medicine, Gunma University, Maebashi 371

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The localization of metallothionein (MT), a heavy metal binding protein of low molecular weight, in the brain of young and old macaca fascicularis was investigated by immunohistochemical technique, and the amounts of MT and heavy metals (Zn, Cu, Cd) were measured by radioimmunoassay and atomic absorption spectrophotometer, respectively. Immunohistochemically, MT was found in pia mater, ependymal cells, protoplasmic astrocytes of the gray matter and fibrous astrocytes of the white matter of cerebrum. Cytoplasm and processes of protoplasmic and fibrous astrocytes showed strong MT immunostaining. Also, vascular feet and adventitia gave positive MT immunostaining. Moreover, both protoplasmic and fibrous astrocytes in pons and spinal cord showed the same positive MT immunostaining as those in cerebrum. In the cerebellum, Bergmann's glias, protoplasmic astrocytes of granular layer and nucleus dentatus, and fibrous astrocytes of white matter showed a strongly positive immunostain for MT.

According to the radioimmunoassay, the amount of MT in the brain of the old one was relatively high-about 19.3 µg/g wet weight. For the heavy metals in the brain, zinc and copper were detected, excepting cadmium.

Metallothionein (MT), an intracellular metalloprotein of low molecular weight (6,000~7,000), has a high content of cystinyl residues (33%) and a high affinity for heavy metal ions such as zinc, copper, cadmium, mercury, silver and platinum (1, 6, 20). It is believed to play physiological roles in the storage and transport of essential metals as well as detoxication of toxic metals (25). Immunohistochemically, the location of MT has been shown not only in the liver and kidney (2, 13, 23), but also in the various organs such as the respiratory epithelial cells of the lung (8), the surface columnar epithelial cells of the intestinal villi (7), the Paneth cells of the small intestine (8), the Sertoli and interstitial cells of the testis (8), and glandular

epithelial cells of the prostate (24). In the present study, we investigated the localization of MT in the brain of macaca fascicularis by immunohistochemistry.

MATERIAL AND METHODS

Preparation of Antibody to Rat Liver MT

Preparation and validation of antibodies against MT conjugated to purified ascaris extract (10) were described in previous reports in detail (11, 15, 23).

Rat MT-I was isolated and purified from the liver of Cd-treated rat according to the method of Tohyama and Shaikh (22). Purified rat MT-I was coupled to polyvinyl pyrrolidone (PVP), obtaining three high molecular weight MTs and inducing high titer of MT antibodies, which subsequently were mixed with

Mailing address: Keiji Suzuki, M. D. Department of Pathology, College of Medical Care and Technology, Gunma University, Showamachi 3-39-15, Maebashi 371, Japan.

Freund's complete adjuvant and emulsified. The emulsions were injected subcutaneously in the backs of the New-Zealand white rabbits six times biweekly. Thereafter, the rabbits were boosted by the intravenous injections of MT-I four times biweekly. The antibody against MT conjugated to ascaris extract was absorbed with ascaris prior to use for MT immunostaining, to avoid an interference of a specific reaction to the substance of ascaris in the tissue with MT immunostaining. Its immunoreactivity to MT in the antiserum was confirmed by immunoblot method after sodium dodecylsulfate-polyacrylamide gel (10~20% gradient gel) electrophoresis (SDS-PAGE); rat MT-1 protein was also detected with silver staining technique after SDS-PAGE.

Histological and Immunohistochemical procedures

The brain and spinal cord of 4 and 16 year-old female macaca fascicularis (crabeating macaque) was immediately fixed in a 10% formalin solution after autopsy. Paraffin sections of cerebrum, cerebellum, pons and spinal cord were prepared for hematoxylin eosin and immunohistochemical staining.

Immunohistochemical localization of MT was detected by anti-MT rabbit serum, using Vectastain avidin-biotinylated horseradish peroxidase complex kit (Vector Laboratories) on paraffin sections.

FIG. 1. Metallothionein protein (lane 2) was stained at single band by silver staining after SDS-PAGE, and after blotting onto nitrocellulose membrane, MT was stained at single band by anti-MT antibody (lane 1). Lane 3: molecular weight standards (kDa).

Analytical Methods

MT in the cerebrum of 16 year-old macaca fascicularis was determined by essentially the same radioimmunoassay as reported earlier (11). For heavy metal analysis, the specimen of the cerebrum was digested with hot concentrated sulfuric acid, and then the contents of zinc, copper and cadmium were measured by atomic absorption spectrophotometer (Perkin Elmer 5000 Atomic Absorption Spectrophotometer).

RESULTS

Purified protein was considered pure, since no additional protein bands could be detected after staining with the silver staining technique (Fig. 1)

Immunoblotting showed antiserum against MT conjugated to ascaris extract, to be specific (Fig. 1).

Absorption test

The use of non-immune rabbit serum and

FIG. 2. Brain of macaca fascicularis shows negative MT immunostaining, using anti-MT serum conjugated with ascaris, after absorption with rabbit liver MT. ABC method. $\times 42$

phosphate buffered saline (pH 7.2) did not result in any specific MT immunostaining in the brain of *macaca fascicularis*. Moreover, the three kinds of anti-MT sera that had been absorbed with rabbit liver MT (Sigma Chemical Co.) did not show a positive MT immunostain in the brain (Fig. 2).

Immunohistochemical findings

Three sera of anti-MT conjugated to ascaris or bovine serum albumin or PVP immunohistochemically showed positive for immunoreactive MT in the brain, and the antibody against MT conjugated with ascaris stained MT of the brain was the best of the three (Fig. 3).

Cerebrum

MT was found in the specific type of cells in the gray and white matter, pia mater, ependyma which lined the ventricles (Fig. 4), and epithelial layer of the choroid plexus.

In the gray matter, the cytoplasm, processes and nuclei of the protoplasmic astrocytes exhibited strong-

ly positive immunostaining for MT (Fig. 5). The processes of these were found around the ganglion cells. The vascular feet of the astrocytes and adventitia of the blood vessels showed positive immunostaining (Fig. 5).

In the white matter, the processes, cytoplasm and nuclei of fibrous astrocytes showed strongly positive immunostaining for MT (Fig. 6). The immunostaining of the fibrous astrocytes was stronger than that of the protoplasmic astrocytes. The vascular feet of the astrocytes and adventitia were also strongly positive to MT immunostaining (Fig. 6).

The astrocytes in the basal ganglia and thalamus were also positive for immunostaining.

Oligodendroglia and microglia showed negative immunostaining for MT in the cerebrum.

Cerebellum

The molecular layers showed fibrillarly or granularly positive immunostaining for MT, and Bergmann's glia in the outermost area of granular layer were positive for MT (Fig. 7). Protoplasmic

FIG. 3. Fibrous astrocytes and vascular adventitia of white matter are positive to MT immunostaining. A contrast between the astrocytes and background is not distinct. A. Stained using anti-MT serum conjugated with bovine serum albumin. B. Stained by using anti-MT serum absorbed to PVP. ABC method. $\times 107$

TABLE 1. *Metallothionein, zinc, copper and cadmium concentration in the cerebrum of macaca fascicularis*

MT	Zn	Cu	Cd
19.3 $\mu\text{g/g}$ wet wt	17.2 $\mu\text{g/g}$ wet wt	6.9 $\mu\text{g/g}$ wet wt	not detected

wt: weight

astrocytes and adventitia of blood vessels in the granular layer, and fibrous astrocytes in the white matter showed a positive MT immunostain. The protoplasmic astrocytes in the nucleus dentatus were strongly stained for immunoreactive MT.

Pons

Protoplasmic astrocytes in the nuclei pontis showed positive MT immunostaining and fibrous astrocytes in the longitudinal tract were also positive (Fig. 8). Processes of fibrous astrocytes were found around the nerve fibers of the longitudinal tract.

FIG. 4. Ependyma is positive for MT-immunostaining using anti-MT serum conjugated with ascaris. ABC method. $\times 214$

Spinal cord

Pia mater, astrocytes in the gray and white matter, and ependymal cells were positive for MT immunostaining.

The findings of MT immunostaining were in the similar areas of the central nervous system of 4 and 16 year-old monkeys.

Analytical Findings

Table 1 summarizes concentration of MT, Zn Cu and Cd in the cerebrum of macaca fascicularis.

DISCUSSION

The antiserum against MT conjugated to ascaris extract which we used in these studies was shown to be specific by immunoblotting.

The antiserum in lanes 1, 2 was indicated at the molecular weight of 14.4 KD which are the polymerized form of MT. This is the normal characteristic of MT; low molecular weight and high in cysteine. It easily polymerizes to be dimers and higher aggregates by oxidative crosslinking (disulfide bond) (16). Furthermore, the liver, kidney and other organs containing MT and stained by this antiserum were reported to be specifically positive (15, 19, 24).

MT was demonstrated in the rat brain (5), and MT-like protein has been identified in the rat brain (9). This study demonstrated the presence of MT by radioimmunoassay, and Zn and Cu by atomic absorption spectrophotometer in the cerebrum of 16 year-old macaca fascicularis.

Immunohistochemically, MT was localized in the protoplasmic astrocytes in the gray matter, fibrous astrocytes in the white matter, adventitia of blood vessels, pia mater, ependyma and epithelial layer of choroid plexus of the brain, but was not found in the oligodendroglia and microglia. MT was found in the nuclei, cytoplasmic process and vascular feet of both protoplasmic and fibrous astrocytes. MT may be involved in detoxification of heavy metals and may also play a role in the storage, transport, absorption, excretion and homeostasis of heavy metals in the tissues (6).

FIG. 5. The processes, cytoplasm and nuclei of protoplasmic astrocytes, vascular foot (small arrow) and blood vessel (large arrows) of gray matter are strongly positive for MT-immunostaining using anti-MT serum conjugated with ascaris. ABC method. $\times 214$

FIG. 6. The processes, cytoplasm and nuclei of fibrous astrocytes, vascular feet (arrows) and vascular adventitia of white matter are strongly positive to MT immunostaining using anti-MT serum conjugated with ascaris. ABC method. $\times 107$
Insert: Nuclei of fibrous astrocytes are positive for MT immunostain without counterstain. ABC method. $\times 429$

FIG. 7. Bergmann's glias and protoplasmic astrocytes in the granular layer of cerebellum are strongly positive to MT-immunostaining using anti-MT serum conjugated with ascaris. P: Purkinje cell. ABC method. $\times 214$

FIG. 8. Fibrous astrocytes in the longitudinal tract (L) and processes of protoplasmic astrocytes in the nucleus pontis (N) are strongly positive to MT-immunostaining using anti-MT serum conjugated with ascaris. ABC method. $\times 214$

Zinc and copper detected in the cerebral tissue by atomic absorption spectrophotometer may be bound to some MT in the neuroglial cells. These cells seem to be an important mediator for the normal metabolism of neurons, because they have vascular feet and their processes are found around the neuron.

MT in the astrocytes may play a role in the

transport of Zn and Cu from blood to neuron, according to this study revealing that the processes of astrocytes were found around the ganglion cells and were attached to the vascular adventitia.

Zinc has an important role in the development of central nervous system, because Zn deficiency induces a severe congenital malformation of the central

nervous system in humans (4). Ohtake and Koga (18) considered that MT was important in the regulation of DNA synthesis through the synthesis or activity of enzymes required for nucleic acid. Nishimura *et al.* (17) have also demonstrated a possible involvement of MT in cell proliferation and differentiation. From these results, MT of the astrocytes may have an important role in the transport of Zn to the brain cells during the development of the central nervous system, and may play a role in the storage and homeostasis of heavy metals after its development.

Immunohistochemically, we found MT localized in nuclei of the astrocytes. The significance of the nuclear MT expression was thought to be the interaction of metal ions with various constituents of the nucleus; they might bind to histons, nucleolar RNA, and nuclear proteins (14).

MT can act as a detoxication site for reactive chemicals (12), and as an efficient free radical scavenger (21). The activities of CuZn-superoxide dismutase were significantly decreased by Zn or Cu deficiency (3). MT in the astrocytes may have important function in the cellular defence mechanism against reactive organic intermediates and free radicals in the brain.

Pia mater showed positive immunostaining for MT. This result suggests that pia mater may have a cerebrospinal fluid-brain function against the heavy metals, because MT plays a role in detoxication of toxic metals.

Ependyma contained MT in the cytoplasm and we found the MT in the epithelial layer in the choroid plexus. The source of cerebrospinal fluid is commonly assumed to be the choroid plexus which secretes the substances in the fluid. The choroid plexus which accompany the ependyma is considered to have a function of Zn, Cu and other heavy metal-homeostasis, and acts as a barrier function against the toxic metals in the brain.

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