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Metallothionein-3 is expressed in the brain and various peripheral organs of the rat

Isao Hozumi[a,*], Junko S. Suzuki[b], Hiroaki Kanazawa[c], Akira Hara[d], Masanao Saio[e], Takashi Inuzuka[a], Shinichi Miyairi[f], Akira Naganuma[g], Chiharu Tohyama[h,b]

[a] Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan
[b] Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba, Japan
[c] Department of Functional Anatomy, University of Shizuoka School of Nursing, Shizuoka, Japan
[d] Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu, Japan
[e] Department of Immunopathology, Gifu University Graduate School of Medicine, Gifu, Japan
[f] Laboratory of Bio-organic Chemistry, College of Pharmacy, Nihon University, Funabashi, Japan
[g] Department of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
[h] Laboratory of Environmental Health Sciences, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Japan

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ABSTRACT

Metallothionein-3 (MT-3), also known as growth inhibitory factor (GIF), was originally identified in the brain. An essential step in elucidating the potential roles of MT-3 is to evaluate its expression levels in organs other than the brain. In this present study, we carried out RT-PCR, Western blot and immunohistochemical analyses to quantify MT-3 mRNA and its protein in the cerebrum, eye, heart, kidney, liver, prostate, testis, tongue, and muscle in male Wistar rats. MT-3 mRNA was detected in the cerebrum, the dorsolateral lobe of the prostate, testis, and tongue. Using a monoclonal anti-MT-3 antibody, we detected MT-3 in the cerebrum, the dorsolateral lobe of the prostate, testis, and tongue as a single band on an immunoblot. Immunohistochemical staining showed MT-3 in some astrocytes in the deep cortex, ependymal cells, and choroidal cells in the cerebrum. MT-3 was also detected in some cells of the glomerulus and the collective tubules in the kidney, some cells in the glandular epithelium of the dorsolateral lobe of the prostate, some Sertoli cells and Lydig cells in the testis, and taste bud cells in the tongue. Although MT-3 immunopositivity was obviously demonstrated in the kidney by the immnunohistochemical method, the expression of MT-3 was not fully detectable by RT-PCR and Western blot analyses. Interestingly, only a subset of cells showed positivity for MT-3, not all cells in all tissues. The localization of MT-3 in peripheral organs outside the brain suggests that MT-3 has roles in these tissues besides its role in growth inhibition of neurites.

Metallothioneins (MTs) are low-molecular-weight, metal-binding proteins that are both self-protective and multifunctional: maintenance of zinc and copper homeostasis, detoxification of cadmium and mercury, regulation of the biosynthesis and activity of zinc-binding proteins such as zinc-dependent transcription factors, protection against reactive oxygen species (ROS), and minimalization of the side effects of chemotherapeutic drugs. MT-3 was first characterized as an inhibitor of an as yet unknown neurotrophic factor in the brain of an Alzheimer's disease (AD) patient and it was first named growth inhibitory factor (GIF) [23]. MT-3 expression level was decreased in AD [20,23] or upregulated following brain injuries such as a stab wound [7]. MT-3-knockout and MT-3-overexpressing mice are very susceptible and resistant to the toxicity of kainic acid, respectively [2]. Although the ectopic overexpression of MT-3 causes pancreatic acinar cell necrosis [16], MT-3 can be protective in other cell types. For example, MT-3 scavenges hydroxyl radicals in cultured neurons and protects fibroblasts under hydrogen peroxide-induced oxidative stress [22]. We previously demonstrated that an adenoviral vector encoding MT-3 cDNA can prevent the degeneration of injured adult rat facial motor neurons [17] and can protect the brain from damage caused by stab wounds in rats [9]. MT-3 has therapeutic potential for neurodegenerative diseases [6,9].

There are several reports on the existence of MT-3 outside the brain [1,3–5,14]. This study is the first in which the existence of MT-3 in various peripheral organs of male rats was examined using three different methods: RT-PCR, Western blot and immunohistochemical analyses. This gives us a new basis for clarifying the specific mechanism of action of MT-3, which may provide considerable information useful for developing treatment strategies using MT-3.

* Corresponding author. Tel.: +81 58 230 6253; fax: +81 58 230 6252.
E-mail address: ihozumi@gifu-u.ac.jp (I. Hozumi).

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Adult Wistar male rats weighing 180–220 g, purchased from CLEA Japan (Tokyo, Japan), were anesthetized with sodium pentobarbital (35 mg/ml). For RNA extraction samples of the following tissues were obtained, frozen in liquid nitrogen, and stored at −80 °C: cerebrum, heart, kidney, liver, prostate (ventral lobe (V) and dorsolateral lobe) (D), testis, tongue (including taste buds), and muscle. An additional set of rats was desanguinated by perfusion through the heart with 0.9% physiological saline (PS) and then the above tissues were dissected out for Western blotting. For the immunohistochemical analysis studies, rats were desanguinated as described and then perfused with a fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.3) and the eyes and the same tissues listed above in addition to the eyes, were dissected out and immersed in the fixative for 8 h. The rats were treated in a humane manner in accordance with the guidelines on the handling of experimental animals issued by the Japanese Association for Laboratory Animal Science.

Total RNA was extracted in accordance with the manufacturer's protocol using Isogen (Nippon Gene, Tokyo, Japan). Template cDNA synthesis was performed using 500 ng of oligo dT (12–18) (Invitrogen, Carlsbad, CA, USA) and 200 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 42 °C for 1 h. PCR-mastermix, containing the specific primers, 10 mM DTT (Invitrogen), 0.5 mM dNPT mix (Invitrogen), 1 unit of RNasin (Promega, Madison, WI, USA), was then added. After inactivation of the enzyme, 4 ng/μl total RNA was applied as PCR samples. The following primer pairs were used: MT-3 sense primer 5′-GCTACAGTCTCTCGCGGCTG-3′ and antisense primer 5′-CATGCTGTCAGGGATTTA-3′. PCR was carried out in a 25-μl reaction mixture containing 0.2 mM of each dNTP and 0.625 units of pfu DNA polymerase (Stratagene, La Jolla, CA, USA). Reaction mixtures were incubated at 95 °C for 2 min, then the reactions were run for 30 cycles, each comprising 15 s at 95 °C, 30 s at 55 °C and 1 min at 75 °C, and final elongation for 7 min at 75 °C. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel, which was stained with ethidium bromide and visualized under UV light.

There are four isoforms of MT; however, only the sequence of MT-3 has a site that can be cleaved by PvuII (Takara,Tokyo,Japan). To confirm the specificity of the MT-3 PCR product, it was digested with 10 units of PvuII in 10 mM Tris–HCl (pH 7.5) containing 1 mM DTT for 1 h at 37 °C. Cleaved products were visualized on a 1.5% agarose gel.

In the preparation for Western blotting, a tissue sample was homogenized in four volumes of 0.01 M PBS (pH 7.4) in a glass-Teflon homogenizer and then the homogenate was then centrifuged for 10 min at 16,000 × *g* at 4 °C. Aliquots of supernatant were collected and centrifugation was repeated twice. The supernatants were applied to centrifugal filter devices (Centricon, YM-30, Millipore, Bedford, MA, USA) and ultracentrifuged for 5 h—overnight at 4,500 × *g* at 4 °C (KR/18,000, Kubota, Osaka, Japan) to collect molecules with molecular weights smaller than 30,000. The filtered aliquots were then centrifuged (VC-960, Taitec, Saitama, Japan) and freeze-dried (VD-600, Taitec, Saitama, Japan) for 1–5 h for concentration. The concentrated aliquots were dissolved in a small amount of 0.01 M PBS and the samples were analyzed for total protein concentration. Ten micrograms of a cerebrum tissue sample and 100 μg of other samples were dissolved in Laemmli's buffer and eletrophoresed on a SDS-PAGE gel with a 5–15% linear gradient under reducing conditions. Proteins were transferred to a polyvinylidine membrane (Immobilon-P, Millipore, Bedford, MA, USA), and then protein blots were blocked in blocking solution (2% skim milk, 0.05 M Tris–HCl, pH 7.6) overnight at room temperature. Blots were incubated with an anti-MT-3 monoclonal antibody, which was previously produced against mouse MT-3 [20], at 1:2,000 in blocking solution for 1 h at room temperature. The blos were incubated with a sheep anti-mouse Ig G horseradish peroxidase-conjugated whole antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2,000 in blocking solution for 1 h at room temperature as the secondary antibody incubation. Immunopositive bands were visualized using ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

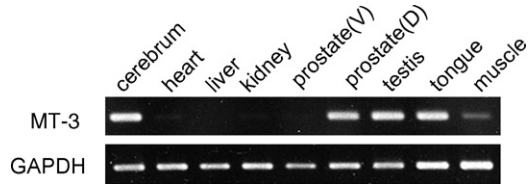

Fig. 1. Detection of MT-3 mRNA in tissues of rats by RT-PCR analysis. The bands are observed clearly for the cerebrum, dorsolateral lobe (D) of the prostate, testis, tongue, faintly for the muscle and more faintly for the heart, kidney, and ventral lobe (V) of the prostate.

In the preparation for immunohistochemistry, dissected tissues were embedded in paraffin blocks and cut into 10-μm thick sections. The sections were immunostained with the same anti-MT-3 antibody used for Western blots using an indirect immunohistochemical technique. Briefly, the sections were deparaffinized and rehydrated. To quench endogenous peroxidase activity, the sections were incubated in 0.3% H_2O_2 in methanol for 10 min. Sections were then washed and blocked in 10% normal goat serum for 30 min. MT-3 was detected with the anti-MT-3 antibody diluted 1:2,000 in PBS overnight in a rocking humidified chamber at room temperature. Primary antibodies were detected by the avidin–biotin complex (ABC) method in accordance with the manufacturer's protocol (Histofine kit, Nichirei Corp., Tokyo, Japan). The sections were then counterstained with hematoxylin. Adjacent sections were stained with hematoxylin–eosin (H&E) or a monoclonal antibody to glial fibrillary acidic protein (GFAP) (1:500, Dako, Glostrup, Denmark) to assess the MT-3-immunopositive regions. The specificity of immunoreaction was confirmed using nonimmunized mouse serum.

As shown in Fig. 1, MT-3 mRNA, was detected clearly in samples derived from the cerebrum, dorsolateral lobe of the prostate, testis, and tongue, faintly in the muscle, and even more faintly in the heart, kidney, and ventral lobe of the prostate by RT-PCR. The MT-3 PCR product (347 bp), which was amplified using the MT-3-specific pimer pairs, contains a PvuII sensitive cleavage site. We also confirmed the sequence of the RT-PCR product from the rat brain using the MT-3-specific primers. All of the PCR products were successfully cleaved into two bands of 219 and 128 bp by PvuII (data not shown). This indicates that the tissues specifically contain MT-3 mRNA.

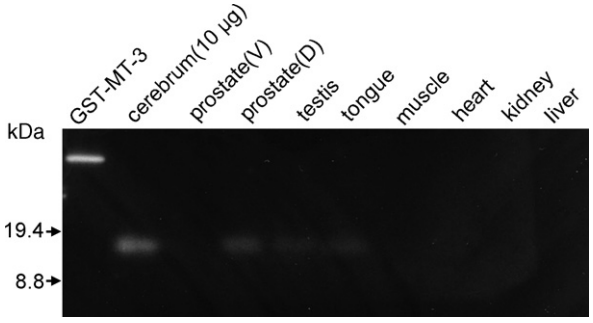

Fig. 2. Detection of MT-3 in tissues of rats by Western blotting using a specific monoclonal antibody against MT-3. 10 μg of total protein of samples of the cerebrum and 100 μg of other tissues were applied to the gel. Bands corresponding to MT-3 mRNA are clearly visible in samples of the cerebrum, dorsolateral lobe (D) of the prostate, testis, and tongue.