— **Minireview** —

Metallothioneins and Neurodegenerative Diseases

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A symposium on the clinical aspects of metallothioneins (MTs) in neurodegenerative diseases was held at the 2003 Society of Metallothionein Meeting in Gifu, Japan. The objectives of the symposium were to review and speculate on the potential roles of MTs, especially MT-3 in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and spinocerebellar degeneration (SCD). Dr. Uchida discussed the controversial problem regarding the expression of MT-3 in AD brains. Dr. Asanuma addressed the function of MTs in the progression of PD and Dr. Hozumi described the therapeutic potential of MTs for ALS, while Dr. Yamada provided immunohistochemical findings of MT-3 in SCD for the first time. Although there are still controversial problems on MTs, this review provides proof that MTs are promising potential therapeutic targets for therapy for some neurodegenerative diseases.

Key words —— metallothionein, growth inhibitory factor, neurodegenerative disease, Alzheimer's disease, amyotrophic lateral sclerosis

Metallothioneins (MTs) are a group of small (6–7 kDa) cysteine-rich (25–30%) metal (zinc and copper)-binding proteins. They are widely and ubiquitously present from molds to humans. Mammalian MTs are thought to be composed of four isoforms (MT-1 to 4), regardless of the presence of additional isoforms. MT-1 and MT-2 are found in all tissues of the body. A similar nucleotide and amino acid sequence makes it difficult to distinguish indisputably by cDNA probe and antibodies (therefore they are abbreviated as MT-1/-2). MT-3 possesses an additional seven amino acids and exists predominantly in the central nervous sytem (CNS). MT-3 was first characterized as an inhibitory substance for unknown neurotrophic factors in Alzheimer's disease (AD).[1] MT-4 is found exclusively in stratified squamous epithelia.[2] MTs are thought to function as self-protective and multifunctional proteins by maintaining zinc and copper homeostasis, detoxifying cadmium and mercury, regulating the biosynthesis and activity of zinc-binding proteins such as zinc-dependent transcription factors, protecting against reactive oxygen species (ROS), and minimizing the side effects of chemotherapeutic drugs. MT-3, which exists mainly in the CNS, has a unique inhibitory effect on neurite outgrowth of cultured neurons that has not been observed for MT-1 and MT-2. The function of MT-3 is very specific and its levels are dramatically reduced in AD brains. Reviews on various basic aspects of metallothionein are available elsewhere.[3–5] In addition, heavy metals such as zinc and copper have been implicated as possible etiological factors in several human neurodegenerative diseases.[6,7] Taken together, these observations suggest that MTs may have important roles in the progress of a number of neurodegenerative diseases such as AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and spinocerebellar degeneration (SCD). This article reviews the roles of MTs, especially MT-3, which were discussed in a symposium on the clinical aspects of MTs in neurodegenerative diseases.

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Table 1. Regulation of MT-3 in Alzheimer's Disease Brain

Method	Region	Stage of AD	Change in MT-III	Authors
Western blot Immunohistochemistry	Frontal cortex (FCx)	moderatesevere	↓	Uchida,1991
Northern blot	Cerebral cortex		↓	Tsuji, 1992
Northern blot Solution hybridization	FCx		no change	Erickson, 1994
RT-PCR	FCx, TCx, Hippocampus		no change	Amoureux, 1997
ELISA Northern blot	Cerebral cortex		↑	Carrasco, 1999
Western blot	TCx		↓	Yu, 2001
	FCx		no change	
Immunohistochemistry	TCx		↓ MT-III (+) cells	
Northern blot	TCx		↓	
GeneChip	CA1	CDR 2-3	↓	Colangelo, 2002

Alzheimer's Disease (AD)

The regulation of MT-3 in AD brain is unique: MT-3 is down-regulated in astrocytes in layers 2 to 6 of gray matter from AD cerebrum,[1)] although MT-1/-2 is up-regulated.[8)] However, the controversial findings on MT-3 regulation in AD brain, reported over the past twelve years (Table 1),[1,9–14)] have generated controversy and confusion. This inconsistency is not based on different analytical procedures (immunoblot or Northern blot). In this section, I discuss the possibility that the contradictory observations, *e.g.* decrease, no alteration, or increase in MT-3 in AD cerebral cortex, may be based on a misunderstanding of the characteristics of neuronal degeneration in AD cerebral cortex.

AD pathology, *e.g.* the accumulation of senile plaques and neurofibrillary tangles (NFTs) and neuronal loss, is not global. Neurodegeneration depends on the region and lamina of the cerebral cortex and on the clinical stage of the disease.[15)] NFT formation and the resultant degeneration first appear in the hippocampus, especially pyramidal neurons in CA1 and layer II of the entorhinal cortex. No neuronal loss or cognitive impairment is observed in individuals at this stage. NFT formation spreads from the limbic system to the neocortex in very mild AD. NFTs distribute to pyramidal neurons in the layer III of inferior temporal cortex, however, they do not reach the superior frontal cortex in this stage. Neuronal loss is present in the hippocampus, but not in the neocortical area. In severe AD, massive NFTs and neuronal loss are found in the hippocampus, and layers III and V of the inferior temporal and superior frontal cortex. The degree of neuronal lopss in the hippocampus and inferior temporal cortex is much greater than that in the superior frontal cortex.

Despite regional and laminar specific pathology in AD brain, less attention has been paid to brain region when quantifying MT-3 protein or mRNA by immunoblot or Northern blot. Immunohistochemical findings revealed that decreased levels of MT-3 protein and mRNA reflect, not all but in part, a laminar pattern of disappearance of MT-3 immunoreactivity in astrocytes, especially in the superficial layer of AD gray matter.[16)] Even in these cases, moderate or strong MT-3 immunoreactivity in astrocytes remains in the deeper layers of the gray matter. Therefore levels of MT-3 protein and mRNA are influenced by the brain area dissected for quantitative analysis. Variations in the level of MT-3 protein or mRNA among AD cases may also reflect the clinical stage of the disease, as the variations in MT-3 level among ALS or CJD cases. Indeed, the intensity of MT-3 immunoreactivity in astrocytes correlates with the number of motoneurons in the ventral horn of ALS patients (Fig. 1). MT-3 immunoreactivity decreases in the cerebral cortex of CJD pa-

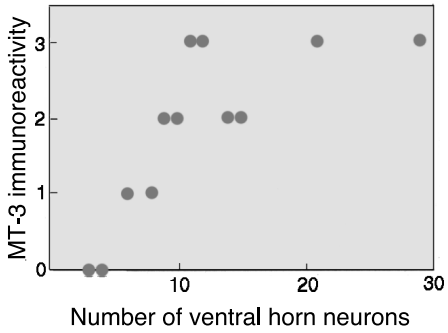

Fig. 1. Representative Relationship between MT-3 Immunoreactivity and Number of Motoneurons in ALS

tients with a long disease duration, but is relatively preserved in CJD with a short disease duration.[17] In AD, the temporal cortex is much more vulnerable than the frontal cortex: neurofibrillary degeneration occurs in the frontal cortex in the late stage of AD. The degree of neuronal loss in the temporal cortex is much greater than that in the frontal cortex, even in severe AD cases. Therefore, it is reasonable that a decrease in MT-3 is more easily found in the temporal cortex than in the frontal cortex.[13]

Thus, MT-3 is down-regulated in degenerative diseases including AD, and an MT-3 reduction is correlated with neuronal loss or disease duration. However, the molecular mechanism of MT-3 down-regulation in neurodegenerative disease is not yet known. *In vitro* studies[18] indicate that the levels of MT-III protein and mRNA are influenced by the state of the cell cycle but not by neuroglial interactions. Neither the cytokines nor growth factors examined down-regulate MT-3 in cultured astrocytes. Although astrocytes in the vicinities of senile plaques (in AD and Down's syndrome) or kuru plaques (CJD) exhibit weak or no MT-3 immunoreactivity, amyloid β peptide (Aβ 1–42) does not down-regulate MT-3 in cultured astrocytes.

Parkinson's Disease (PD)

Down-regulation of MT-3 expression has been reported in astrocytes in the brain of PD.[18,19] MTs exert antioxidant effects by scavenging reactive oxygen species (ROS) to protect against oxidative stress-induced cell damage.[20–23] The formation of ROS and dopamine quinones plays an important role in the pathogenesis of PD that is characterized by dopamine depletion in the basal ganglia and dopaminergic cell death in the substantia nigra pars compacta.[24–27] We have reported that levodopa, a therapeutic drug for PD, may be a source of ROS which enhances lipid peroxidation and cell damage, and that long-term treatment with levodopa may accelerate various adverse effects and the progression of PD in certain patients.[26,28] Dopamine increased MT-3 mRNA expression through the generation of ROS in a glial cell line.[29]

Expression of MT-3 mRNA was examined in the basal ganglia of hemi-parkinsonian rats lesioned by 6-hydroxydopamine (6-OHDA) in order to clarify the changes in MT-3 expression and its regulation of levodopa in dopaminergic neurodegeneration.[30] In normal rats, levodopa/carbidopa (25/2.5 and 50/5 mg/kg, i.p.) significantly increased the expression of striatal MT-3 mRNA in a dose–dependent manner 24 hr after the administration. The induction of MT-3 mRNA showed a peak at 24 hr after levodopa/carbidopa administration. The levodopa-induced MT-3 mRNA expression might represent a complementary reaction against oxidative stress. In experiments in hemi-parkinsonian rats, MT-3 mRNA expression was significantly decreased on the 6-OHDA-lesioned side in the striatum 24 hr after vehicle treatment. On the 6-OHDA-lesioned side, the levodopa/carbidopa (25/2.5 mg/kg, i.p.) treatment showed no increase in the expression of MT-3 mRNA. These results suggest that free radical scavenging potency including MT-3 is reduced in the parkinsonian brain, and that levodopa fails to induce MT-3 mRNA expression to consequently accelerate the progression of PD.

Using MT-1/-2 knock-out (KO) mice, Penkowa *et al*. showed that MTs play regulatory roles in both inflammatory and regenerative responses to injury in the brain.[31,32] The effects of MT-1/-2 on the dopaminergic neurotoxicity of 6-OHDA were examined in MT-1/-2 KO mice by intracerebroventricular injection of 6-OHDA.[33] The loss of dopamine neurons in the substantia nigra induced by the 6-OHDA intracerebroventricular injection was significantly aggravated in the MT-1/-2 KO mice. This suggests that MT-1/-2 exerts neuroprotective effects against the dopaminergic neurotoxicity of 6-OHDA at the nigral cell body by scavenging free radicals.

Aging is a common background of both AD and PD. MT-3 and its mRNA expression in the brain of endotoxin LPS-treated aged rats (24 months old) was examined to elucidate the age-related changes in MT-3 expression and its inducibility against oxidative stress.[34] In the frontal cortex of aged rats, the basal expression of MT-3 mRNA was significantly increased. In the frontal cortex and thalamus + midbrain of young adult rats, the level of MT-3 mRNA

was increased 2 hr after the LPS administration (20 mg/kg, i.p.), but not in the aged rat brain with LPS administration. MT-3 immunopositive cells were increased in the frontal, parietal, and piriform cortices, hypothalamus, and amygdaloid nucleus with aging. The LPS treatment induced MT-3 expression in neurons and astrocytes, especially in oligodendrocytes and microglia in these brain regions of young adult rats at 6 hr after the treatment, but not in glial cells in the aged rat brain. The induction was observed in neurons and astrocytes, especially in oligodendrocytes and microglia by oxidative stress. The reduced induction of MT-3 in aged rats by oxidative stress may be a background factor in the pathogenesis of aging-related neurodegenerative diseases such as PD.

Amyotrophic Lateral Sclerosis (ALS)

ALS is a progressive lethal neurodegenerative disease. Patients with ALS show progressive muscle weakness and atrophy, dysphagia, and dyspnea. The pathological findings show selective loss of motor neurons in the spinal cord, brain stem, and cerebral motor cortex, accompanied by gliosis. About 10% of cases are familial ALS (FALS) and 10–25% of FALS is caused by mutations in the gene for copper-zinc superoxide dismutase (SOD1).[35–37] As these cases are clinically and pathologically indistinguishable from sporadic ALS (SALS) except for the earlier age of onset, the observation suggests a common pathogenetic mechanism is shared with ALS. SOD1 is a metalloenzyme with active sites for both copper and zinc. SOD1 detoxifies the superoxide anion to hydrogen peroxide, which in turn is converted to water. However, the reduced enzymatic activity of SOD1 cannot simply explain the pathogenic mechanism of ALS. The loss of motor neurons is not thought to be due to the reduction in normal SOD1 activity, but rather to some toxic gain of function of the mutated SOD1 protein. The exact pathophysiological mechanism for ALS remains unknown and has been discussed elsewhere.[38,39]

In 2002, three intriguing studies on ALS were reported. First, the crossing of ALS model mice (G93A SOD1) with MT-1/MT-2 or MT-3 knock-out mice was found to reduce survival time and accelerate the onset and progression of ALS, respectively.[40] Previously, Nagano *et al*. reported that FALS mice reach the onset of clinical signs and death significantly earlier in response to a reduction in MT-1/-2 expression.[41] Secondly, Igarashi *et al*. analysed SALS spinal cords using molecular indexing combined with cDNA microarray and found that the expression levels of six genes were altered. One was MT-3 mRNA, which is decreased in SALS spinal cords.[42] On the other hand, another report found a somewhat higher level of MT-3 mRNA in SALS.[43] The elevation of MT-3 expression in G93A SOD1 transgenic mice has been reported.[44,45] For further evaluation, we believe that the time course and the area dissected for analysis should be considered with the pathological findings of each case. Thirdly, Uchida *et al*. reported MT-3 is strongly potent at scavenging hydroxyl radicals.[46]

Our preliminary evaluation of an immunohistological examination on normal and SALS spinal cords using a rabbit serum against human MT-3 (provided by Dr. Y. Uchida) showed that the immunoreactivity of MT-3 in astocytes was markedly reduced in cases of SALS epecially with respirators (Yamada M. *et al*., in preparation). Some other factors that may influence the expression of MT-3 in cases with ALS should be considered for further evaluation.

Recently we have reported that an adenoviral vector encoding rat MT-3 cDNA (AxCArGIFM) prevented the loss of facial motor neurons after facial nerve avulsion.[47] Avulsion of cranial and spinal nerves in rats causes marked degeneration of motor neurons, making the animal model useful for therapeutic evaluation of neurotrophic factors or neuroprotective molecules against motor neuron injury. The treatment with AxCArGIFM after avulsion significantly prevented the loss of injured facial motor neurons, improved choline acetyltransferase immunoreactivity and prevented induction of nitric oxide synthase activity in the motor neurons. This indicates that MT-3 may have therapeutic potential against motor neuron degeneration and motor neuron injury.

Taken together with the finding that MT has multiple functions which maintain zinc and copper homeostasis, detoxify heavy metals such as cadmium and mercury, and protect against reactive oxygen species (ROS), MT, especially MT-3, may be a promising therapeutic agent for ALS. We propose possible roles for MT in the mechanism of progression of ALS in Fig. 2.

Spinocerebellar Degeneration (SCD)

We investigated the expression profiles of MT-3 in cases with neurodegenerative diseases, especially SCD. Formalin-fixed, paraffin-embedded sections were obtained from autopsy brains and immunostained with rabbit serum against human

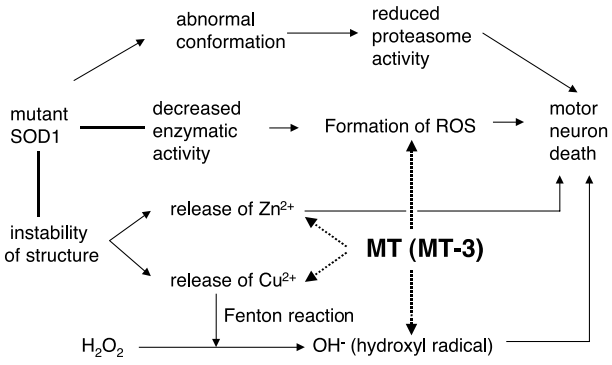

Fig. 2. Schematic Representation of Proposed Mechanism of ALS Associated with MT

Dotted lines show presumed therapeutic functions of MT, especially MT-3, in the progression of ALS.

MT-3 (provided by Dr. Y. Uchida).

In normal human brains, MT-3 was mainly expressed in astrocytes in both the gray and white matter. The expression was observed throughout the CNS, showing similar staining intensity in most of the brain regions; however, labeling was relatively weak in several regions such as the pontine nuclei, inferior olive, and medial nuclei of the thalamus. In contrast, the cerebellar dentate nucleus showed relatively intense labeling.

In progressive supranuclear palsy (PSP), the cerebellar dentate nucleus showed moderate to severe loss of neurons, and MT-3 expression was diffusely decreased. In multiple system atrophy (MSA), the pontine nuclei showed severe loss of neurons and low-level expression of MT-3 in reactive astrocytes. The cerebellar cortex exhibited loss of Purkinje cells and a decrease in MT-3-expression in Bergmann glia. These results suggest that MT-3 is down-regulated in the affected regions in the brain.

We also investigated the brains of CAG-repeat diseases. In Machado-Joseph disease (MJD), the pontine nuclei are known as a moderately affected site, and labeling of MT-3 in astrocytes was weak, like control cases. In the pontine nuclei of a MJD patient, who died in an early stage of the disease due to hepatic failure, however, MT-3 was highly expressed in astrocytes in spite of preservation of the neuronal population (Fig. 3). In dentatorubral-pallidoluysian atrophy (DRPLA), we investigated brains of all three phenotypes, juvenile, early adult, and late adult types, according to Naito's classification.[48] It has been reported that in the cerebral cortex of DRPLA brains, no apparent neuronal loss is detected, but mutant DRPLA proteins with expanded polyglutamine stretches are extensively accumulated in neuronal nuclei.[49] In the cerebral cortex of DRPLA, MT-3 labeling was increased in the juvenile type (Fig. 4), but was relatively decreased in the late adult type. It is also known that DRPLA patients present with white matter lesions, and that glial cells including oligodendrocytes are involved in the pathogenesis of the disease. In the affected white matter, MT-3 expression in astrocytes was increased, more intensely in the late adult type. Huntington's disease (HD) is one of the polyglutamine diseases and shows neuronal loss in the frontal lobe and striatum, especially in the caudate nucleus. The degree of dementia in this disorder does not correlate well with the severity of cortical neuronal loss. Recent studies have indicated that mutant huntingtins, the causative gene products, are accumulated in cell nuclei of many remaining neurons in the affected regions.[50] Intense labeling of MT-3 was observed in many astrocytes in these lesions, especially in the caudate nucleus. In addition, we investigated brains afflicted by corticobasal degeneration (CBD), in which the frontal lobe demonstrated moderate loss of cortical neurons. MT-3 immunohistochemistry showed intense labeling of astrocytes in the affected cortex (Fig. 5), as well as in the subcortical white matter.

The observations show that MT-3 is down-regulated in the affected brain regions in neurodegenerative diseases such as PSP, MSA, and AD. However, interestingly MT-3 expression is up-regulated in astrocytes in some types of neurodegenerative diseases such as CBD, probably in response to neuronal early dysfunction caused by specific pathogenesis. The expression of MT-3 may be differently affected by the mechanisms and stages of the diseases. The reduced expression of MT-3 in astrocytes in some cases with SND does not seem to be necessarily associated with the intensity of neuronal loss.

Concluding Remarks

There is still controversy with respect to the expression of MT-3 in AD. A similar problem exists in research on ALS. The area dissected for analysis, time course and in addition some factors which influence the expression of MT-3 should be considered for further evaluation. In each neurodegenerative disease, some specific factors may influence MT-3 expression. There is also some controversy regarding the cellular localization and functions of

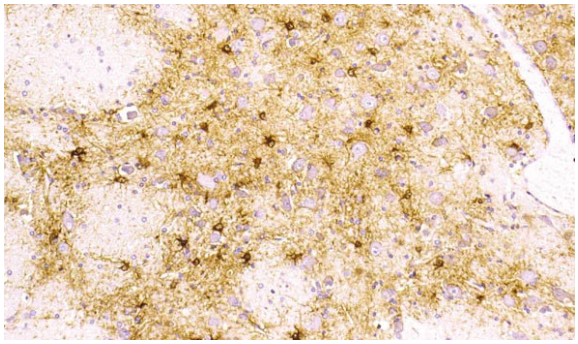

Fig. 3. MT-3 Immunohistochemistry in the Pontine Nuclei of a Patient with MJD
Intense labeling is present in astrocytes.

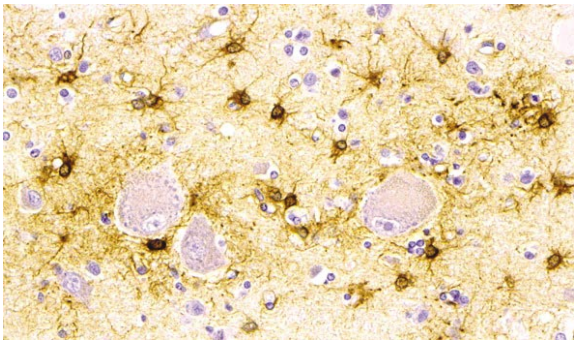

Fig. 5. MT-3 Immunohistochemistry in the Cerebral Cortex of a Patient with CBD
Intense labeling is present in astrocytes.

Fig. 4. MT-3 Immunohistochemistry in the Cerebral Cortex of a Patient with DRPLA
Intense labeling is present throughout the cortex, being localized in astrocytes.

MT-3. MTs are multifunctional. MT-1/-2 are considered to be acute phase proteins. The induction and changes seems to be more dramatic than those for MT-3. MTs are able to prevent the formation of free radicals. MT-3 may provide long-lasting neuronal protection. Penkowa *et al*. proposed that MT-2A has significant potential as a therapeutic drug for multiple sclerosis.[51] Neurodegenerative diseases, especially ALS, are associated with oxidative stress. Neurodegenerative diseases may share a converging pathogenetic mechanism which can be a target for the pharmacological therapy. Taken together, the observations and discussion provide proof that MTs, especially MT-3, may be promising pharmacological candidates for therapy in some neurodegenerative diseases.

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