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Review Article

Metallothioneins and Brain Injury: What Transgenic Mice Tell Us

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Abstract

In rodents, the metallothionein (MT) family is composed of four members, MT-1 to MT-4. MT-1&2 are expressed in virtually all tissues including those of the Central Nervous System (CNS), while MT-3 (also called Growth Inhibitory Factor) and MT-4 are expressed prominently in the brain and in keratinizing epithelia, respectively. For the understanding of the physiological functions of these proteins in the brain, the use of transgenic mice has provided essential information. Results obtained in *MT-1&2*-null mice and in MT-1-overexpressing mice strongly suggest that these MT isoforms are important antioxidant, anti-inflammatory and antiapoptotic proteins in the brain. Results in *MT-3*-null mice show a very different pattern, with no support for MT-1&2-like functions. Rather, MT-3 could be involved in neuronal sprouting and survival. Results obtained in a model of peripheral nervous system injury also suggest that MT-3 could be involved in the control of nerve growth.

Key words: metallothionein, transgenic mice, cytokines, brain injury, oxidative stress

Introduction

Almost half a century ago, an unusual cadmium-binding protein was isolated from the horse kidney (1). Due to its high content of metals and cysteine residues, this protein was named metallothionein (MT) (2, 3). Over the years, an exponential number of reports have been published concerning structural, biochemical, regulatory and physiological aspects of MT (4–9). In the last 10–15 years, however, MT research in the brain has provided a significant insight on the putative physiological functions of these proteins, in part because transgenic mice have been extensively used.

On the basis of structural relationships, MTs have been subdivided into classes or families (10). Four closely linked *MT* genes (*MT-1-4*) are present in rodents (11, 12), while several *MT-1* gene variants are present in ungulates and primates (4, 13, 14). MT-1 and MT-2 (MT-1&2) are expressed coordinately in most tissues including those of the central nervous system (CNS) (15–17), while MT-3 and MT-4 show a much more restricted tissue expression (basically in the CNS and stratified squamous epithelia, respectively).

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Metallothionein-1&2 are preferentially expressed in reactive astrocytes

MT-1&2 expression in the brain has been demonstrated in many species including the mouse (18), human (19), monkey (20), dog (21), sheep (22), and cow (23). Studies of *in situ* hybridization (21, 24–28) and immunohistochemistry (19, 21, 28–38), have demonstrated that MT-1&2 expression occurs throughout the brain and spinal cord, and that the principal cellular source is the astrocyte, particularly following injury. Significant MT-1&2 expression is also found in ependymal cells, epithelial cells of the choroid plexus, meningeal cells of the pia mater and endothelial cells of blood vessels, while neurons appear to express very low levels. Although in the normal brain the microglia are devoid of MT-1&2, these cells upregulate MT-1&2 expression in response to injury (see (39) for further discussion).

Expression of metallothionein-3 in the Central Nervous System

The identification of MT-3 by Uchida and co-workers (40) was a major breakthrough in the MT field. The protein was discovered in the human brain in the context of the neurotrophic unbalance hypothesis related to Alzheimer disease (AD). Because of its activity in a bioassay in which the survival of rat neonatal cortical neurons was examined, MT-3 was originally named Growth Inhibitory Factor (GIF). A significant decrease in MT-3/GIF was observed in AD brains, and therefore it was suggested that this protein had an underlying involvement in the neuropathology of this devastating disease.

In contrast to MT-1&2, the expression of MT-3 is much more restricted. Initially regarded as a CNS-specific isoform (11, 40–42), it is now clear that MT-3 is expressed in other tissues, in some cases in a development-dependent manner (43–49). In addition to human and mouse brain, MT-3 has been reported in other species such as horse and cow (50), rat (51), pig (52), dog (53), and sheep (54).

There is still significant uncertainty regarding the cellular source of MT-3 in the CNS. Using antibodies raised against the unique 6-amino acid insert of MT-3/GIF, *in situ* hybridization, or Northern blot analysis, Uchida et al. (40, 42, 55) observed that MT-3 expression was prominent in astrocytes but not in neurons or other brain cells. However, many other reports have not supported an astrocyte-specific MT-3 expression. Although in most cases no thorough cell identification has been established by robust methods such as double labeling or use of the MT-3 KO mice as controls, in the normal brain it appears that the MT-3 mRNA signal is more consistent with neuronal than with glial cells, with astrocyte MT-3 upregulation eventually occurring following injury (26, 27, 36, 53, 56–68). In contrast to the MT-3 messenger, the MT-3 protein has been shown to be present mainly in astrocytes by a number of studies (36, 40, 65, 66, 69–74). Other reports, however, show MT-3 protein presence basically in neurons (63, 64, 75–77). One report demonstrated MT-3 protein not only in neurons, astrocytes and microglia, but also in oligodendrocytes, following lipopolysaccharide (LPS) injection (78). A thorough analysis of the antibodies used in these reports strongly suggests that the cellular source of MT-3 depends heavily on the antibody used. Whether this reflects a problem of specificity of the antibody or a more biological problem (putative neuronal MT-3 secretion and astrocyte uptake of the protein) is currently unknown and deserves further work.

Transgenic mice show that metallothionein-1&2 are essential proteins for coping with brain damage

There is compelling evidence that MT-1&2 are involved in the response of the brain to damage. Thus, significant upregulation of these proteins has been observed in a number of human neurological diseases, including Alzheimer's disease (55, 79–82), Pick's disease (79), short-course Creutzfeld-Jakob disease (72), amyotrophic lateral sclerosis (83–85), and multiple sclerosis (86, 87). Experiments carried out in animal models fully demonstrated the response of MT-1&2 to brain damage elicited by inflammatory factors such as lipopolysaccharides (11, 15, 24, 88), stress (62, 89–91), glutamate analogues (37, 51, 59, 92–95), cryogenic injury (28, 32, 66, 71), stroke/ischemia (17, 95–98), familial amyotrophic lateral sclerosis models (38, 67, 99, 100), multiple sclerosis models (101–103), and gliotoxins (104–106).

In both human neurological diseases and animal models of brain injury, cytokines and/or oxidative stress are likely to be involved (107–111). That cytokines may cause significant brain damage has been clearly demonstrated by results obtained in transgenic mice expressing in astrocytes cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-3 (IL-3) or interferon-α (IFN-α) under the control of the glial fibrillary acidic protein (GFAP) gene promoter. Each of these transgenic mice showed a specific phenotype of cytokine-induced damage that resembled some of the most important neurological diseases in humans (112, 113). As expected from the above studies, a dramatic upregulation of MT-1&2 was observed in clinically symptomatic GFAP-IL6 (27, 114), GFAP-TNF (36), GFAP-IL3 and GFAP-IFNα (115) mice. In some of these mice it has clearly been demonstrated that oxidative stress is increased, and since MT-1&2 are potent antioxidant proteins and are induced by oxidative stress (7, 116, 117), it is likely that this cytokine-driven MT-1&2 expression is at least in part related to tissue injury rather than to a specific gene regulation. Nevertheless, data obtained in IL-6-null mice have supported a specific role of IL-6 in MT-1&2 gene regulation (62, 66, 118).

Increased MT-1&2 expression following tissue injury does not necessarily indicate an important role. The generation of genetically modified mice (119–121) has undoubtedly demonstrated that these proteins are indeed important in the CNS. Thus, mice overexpressing MT-1 were partially protected against mild focal cerebral ischemia and reperfusion, showing lower infarcts and better functional recovery than the controls (97). In accordance, the opposite was observed in MT-1&2-null mice (98), pointing to an essential role of MT-1&2 in coping with ischemic damage of the brain. Other studies with transgenic mice have equally involved MT-1&2 as important proteins following damage elicited by kainic acid-induced seizures (94), gliotoxins (105, 122), 6-hydroxydopamine (123), amyotrophic lateral sclerosis (38, 100), multiple sclerosis (103, 124), traumatic brain injury (28, 125, 126), and transgenic IL-6-induced neuropathology (127–129). All of the above are presumably quite different models of brain injury, yet the general impression is that MT-1&2 have similar effects in all cases, namely, decreasing oxidative stress, inflammation and apoptosis in the CNS. Although is out of the scope of this review, there is compelling evidence of the antioxidant roles of these proteins in many other tissues as well, albeit the exact mechanisms underlying these effects are still under debate. The quenching of free radicals, metal (Zn, Cu) events, effects on the cell redox status or changes in gene expression are among the possible scenarios for a more detailed analysis.

Exciting perspectives are opened by the fact that exogenously applied MT-2 mimicked MT-1 transgenic overexpression, causing a significant clinical improvement in an animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (101, 130), and protecting against traumatic brain injury (126, 131), calling for the therapeutic use of these proteins. It is still unknown whether the MT protein acts extracellularly or is incorporated somehow into the cell, but considering the potential importance of this result, further work in this regard is welcome.

Transgenic mice show different metallothionein-3 functions

The expression of MT-3 is affected by a number of human neurological diseases. Although MT-3 was initially reported to be downregulated during AD, this is unfortunately not a consistent finding (40, 41, 71, 76, 132–134). Whether this has to do with methodological problems (as discussed above, different antibodies give different results) or different AD etiologies is currently unclear. MT-3 expression has been shown to be altered (up- or downregulated) in Down syndrome (135), Creutzfeld-

Jakob disease (72), pontosubicular necrosis (73), Parkinson disease, meningitis, and amyotrophic lateral sclerosis (134). A similar trend (up- or downregulation depending on the model, time, etc.) has been observed in animal models of brain injury. Thus, MT-3 expression has been increased by stab wounds (56, 70, 136) and kainic acid administration (56), but decreased by cortical ablation of the somatosensory cortex (57), facial nerve transection (58), and middle cerebral artery occlusion (137). A biphasic response of MT-3 to CNS injury, with initial downregulation followed by upregulation, was observed in response to N-methyl-D-aspartate (NMDA) (65) or to a cryolesion (66, 125).

As described above for MT-1&2, the generation of genetically modified mice will also help significantly in the understanding of the potential biological roles of MT-3 (138, 139). In normal conditions, MT-3-null mice do not show any appreciable phenotype other than a higher expression of GFAP in old animals compared to that in wild-type mice (139). Following kainic acid-induced seizures, these mice show enhanced sensitivity, convulsing longer and having greater mortality than littermate controls. A thorough analyses of their brains revealed increased neuronal death in the CA3 pyramidal cell layer of the hippocampus, as could be expected, although such effect was limited to the low-convulsing animals. In accordance with these results, transgenic mice overexpressing MT-3 showed the opposite trends (139). These *in vivo* results strongly suggest that MT-3 has a neuroprotective role. In line with this, *in vitro* work has also demonstrated neuronal protection against glutamate neurotoxicity, presumably affording protection against nitric oxide (140, 141). Moreover, the neuroprotective role of MT-3 against toxicity caused by β-amyloid 25–35 peptide (142–144), *S*-nitroso-thiols and H_2O_2 (141), and high oxygen conditions (145) has also been demonstrated.

Despite much discrepancy regarding the exact mechanisms involved (i.e., (145) *vs.* (141, 146)), the impression that MT-3 may be acting as an antioxidant protein is mounting, likely involving significant differences with its MT-1&2 counterparts (147). Nevertheless, the results obtained with MT-3-null mice call for caution. While increased neuronal death in the CA3 area following kainic acid administration (139) has been confirmed (77), the same study also showed decreased neuronal death in the CA1 area, thus indicating a protective or detrimental role of MT-3 depending on the brain area, at least in the kainic acid model. In another transgenic model of brain injury, transgenic G93A SOD1 mice, in which mutated superoxide dismutase (mSOD1) shows abnormalities in zinc binding and has enhanced nitration activity and thus oxidative damage ((100) and references therein), the role of MT-3 was studied by crossing the G93A SOD1 mice with MT-3-null mice. The results clearly demonstrated that the deficiency of MT-3 potentiated motor neuron impairment as revealed by stride length and grip strength, likely because of increased motor neuron death. In line with this study, a recent report showed that an adenoviral vector encoding rat MT-3 prevents the degeneration of injured motoneurons (148). However, MT-3 deficiency potentiated motor neuron death in early symptomatic but not in end-stage G93A SOD1 animals, indicating the limited protection afforded by MT-3. This is essentially what has also been observed in the kainic acid-induced seizure model (139). In the cryolesion model, MT-3 deficiency did not affect the inflammatory response, oxidative stress and apoptotic death (149), in sharp contrast to what is observed in MT-1&2-deficient mice (see above). Whether this indicates that MT-3 does not function effectively as an antioxidant protein *in vivo* in this certainly highly damaging model, or if it again highlights the limited protection afforded by this protein, remains to be established. MT-3 deficiency did increase the expression of some neurotrophins and other factors that may significantly affect neuronal survival and/or growth, such as growth-associated protein-43 (GAP-43) (150, 151), which would support an inhibitory role of MT-3 in line with some *in vitro* bioassays (40, 131, 132, 152–154). A recent report analyzing the response of MT-3-deficient mice in a peripheral nerve model supported the role of MT-3 as an inhibitory factor of neuronal sprouting (155), since axonal regeneration was faster, as substantiated electrophysiologically and histologically. Thus, it might be envisaged that MT-3 serves different functions in the CNS, promoting neuronal survival or death depending on the type of cells and/or brain area, while inhibiting neuronal sprouting. Further work on this fascinating family of proteins is warranted.

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