Tohoku J. Exp. Med., 2002, **196**, 9–22

Review

Recent Studies on Metallothionein: Protection Against Toxicity of Heavy Metals and Oxygen Free Radicals

MASAO SATO and MASUO KONDOH

Department of Public Health, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770–8514

SATO, M. and KONDOH, M. *Recent Studies on Metallothionein: Protection Against Toxicity of Heavy Metals and Oxygen Free Radicals.* Tohoku J. Exp. Med., 2002, **196**(1), 9–22 —— Metallothionein (MT) is a ubiquitous, cysteine-rich, metal-binding protein. MT synthesis is induced by various stimuli such as cadmium, mercury, zinc, oxidative stress, glucocorticoid, and anticancer agents. Recently, transgenic mice with loss-of-function mutations in the MT-I/-II genes were established. It has been assumed that MT plays a role in the detoxification of heavy metals. In recent studies using MT-null mice, the ability of MT to protect against cadmium-induced renal, liver and bone injuries has been confirmed. Moreover, MT is also capable of scavenging oxygen free radicals. MT is involved in the protection of tissues against various forms of oxidative injury, including radiation, lipid peroxidation, oxidative stress caused by anticancer drugs, and conditions of hyperoxia. However, MT still lacks an established biological function. Unexpectedly, the MT-null mice were apparently in good health, and the critical biological roles of MT have been questioned. MT seems to be a protective protein produced in response to a variety of stresses. Here, current studies on the protective roles of MT against toxicity of heavy metals and reactive oxygen species are reviewed, and the putative biological functions of MT are discussed. ———— metallothionein; cadmium; zinc; reactive oxygen species; mitochondria

The human body, which is composed of 60 trillion cells, is constantly exposed to environmental stress, and functions by extensively operating a complex and diverse information network and mobilizing relevant anti-stress systems, there by maintaining homeostasis. Metallothionein (MT) has attracted attention as a protein possibly related to metabolism and

Received December 28, 2002; revision accepted for publication January 25, 2002.

Address for reprints: Dr. Masao Sato, Department of Public Health, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, 180 Yamashiro-cho, Tokushima 770–8514, Japan.

e-mail: msato@ph.bunri-u.ac.jp

This paper was presented at the Sendai Symposium: Paradigm Shift in Heavy Metal Toxicology held on November 21–22, 2001.

detoxification of heavy metals because it has the lowest affinity to the essential metal zinc but a high affinity to toxic heavy metals such as Cd and Hg (Kagi and Vallee 1960). Also, because its expression is induced by a wide range of stressors including not only chemical stressors, such as heavy metals and activated oxygen generators, but also physical stressors, such as restraint, it is considered to have a preventive effect against the disruption of homeostasis due to environmental stress. At first, the physical functions of MT were discussed phenomenologically, but the advent of the MT-null mouse in 1993 and 1994 triggered rapid progress in research on the relationships between biological phenomena and MT (Michalska and Choo 1993; Masters et al. 1994). In addition, major paradigm shifts have occurred in the fields of bioscience due to the recent explosive development of genome science, and studies on MT are expanding in the swells of this trend.

In this review, therefore, papers that present new subjects in MT studies are selected from those published over the past few years after our previous work (Sato 1992; Sato and Bremner 1993; Sato 1994) and reevaluated to trace the course of the paradigm shift in MT studies.

Chemical properties

MT is a low-molecular-weight protein with a molecular weight of approximately 6000 discovered in 1957 in the equine kidney (Margoshes and Vallee 1957). It is a cysteine-containing protein in which about one-third of the constituent amino acids are cysteine and no aromatic amino acid is contained. Thus, it has many intramolecular SH groups that are considered to be present in the free form, but has no intramolecular S-S bond. Cysteine residues are considered to play central roles in the diverse physiological functions of MT such as heavy metal detoxification, metal metabolism regulation, and radical scavenging action (Sato 1992; Sato and Bremner 1993; Davis and Cousins 2000; Kondoh and Sato 2002). In fact, Zn and Cd are captured in the MT molecule by the SH groups. Each molecule of MT can bind seven molecules of Zn and Cd by forming two clusters (α cluster and β cluster). Interestingly, there is a rule for the uptake of metals in the two clusters: After four molecules have been taken in by α cluster, three metal molecules are captured in β cluster (Andrews 1990). These observations suggest differences in the physiological functions between the two clusters. In fact, recent evaluation has revealed that Zn in β cluster is involved in the regulation of mitochondrial oxygen respiration by MT (Ye et al. 2001), which is of great interest in the evaluation of the physiological functions of MT. The degree of affinity of MT to metals is Zn$<$Cd$<$Cu$<$Hg, Ag (Kagi and Vallee 1960). Although all 20 thiol groups present in MT are usually coordinated with metal, the ability of MT to capture hydroxyl radicals, which are primarily responsible for the toxicity, is more than 300 times greater than that of glutathione (Sato 1992).

There are four types of metallothioneins, namely, I, II, III, and IV isoforms in mammalian MT, but the positions of cysteine are the same among these isoforms. Generally, MT means MT-I and MT-II isoforms. MT-I and MT-II are expressed in nearly all organs of the body, and are induced by a wide variety of stressors including heavy metals and oxidative stress-inducing agents. MT-III is an MT isoform isolated based on its inhibitory effect on neuron proliferation, and its expression is localized in the brain (Uchida et al. 1991). However, because cell proliferation was suppressed in the pancreas and hamster kidney cells when MT-III was allowed to express (Palmiter 1995; Quaife et al. 1998), MT-III is considered to non-specifically suppress cell proliferation. MT-IV was isolated as a gene induced in the differentiation of stratified squamous cells and is expressed specifically in epithelial cells of organs such as the tongue and skin (Quaife et al. 1994).

Regulation of metal metabolism

MT is a metal-binding protein present in abundance in cells and was isolated from tissues as complexes with Zn. Among the isoforms of MT, type I and type II are expressed in most cultured cell lines, cell types, organs, and tissues, and are considered to have evolved as a means to maintain Zn homeostasis (Andrews 2000). Also, because MT is induced by the administration of Zn or Cd, it was considered to be involved in heavy metal metabolism. However, whether MT is involved in the uptake of metals by tissues or not has remained unknown.

Concerning the Zn metabolism regulating effect of MT, many evaluations have been performed using MT-null mice and MT-overexpressing (MT-Tg) mice. Zn is accumulated in the liver during inflammation. Not only is Zn involved in the activities of more than 300 enzymes, it also regulates the gene expression via the zinc finger, and the mechanism of Zn accumulation in inflammatory reaction has attracted attention. The increase in hepatic Zn concentration after the administration of lipopolysaccharide, was observed in normal mice but not in MT-null mice. Therefore, MT was suggested to be responsible for Zn accumulation during inflammatory reaction (Philcox et al. 1995). Also, urinary Zn excretion levels measured during a fast or Zn intake restriction was increased in MT-null mice compared with normal mice (Philcox et al. 2000). These results suggest that MT has the ability to retain Zn under physiological conditions. Also, in MT-Tg mice, Zn was accumulated in female organs, and the teratogenicity of Zn deficiency during pregnancy was significantly alleviated (Dalton et al. 1996). On the other hand, tissue Zn concentration was reduced, and the sensitivity to Zn deficiency during pregnancy was enhanced in MT-null mice (Andrews and Geister 1999). Moreover, Zn deficiency caused abnormalities in neonate kidney differentiation in MT-null mice (Kelly et al. 1996). From these findings, MT is considered to have a Zn metabolizing activity in the individual level. However, as MT-null mice are born in a normal state, MT is unlikely to be essential for Zn metabolism in the normal state; it is considered to be important for the regulation of Zn metabolism under stress.

The uptakes of Cd by the liver, kidney, and pancreas early after Cd administration were similar between MT-null mice and wild-type mice; therefore MT is not involved in the tissue uptake of Cd (Liu et al. 1996). However, the Cd concentration markedly decreased after reaching the peak in MT-null mice, indicating that MT causes Cd retention in tissues. No such tendency was noted in the digestive organs.

Alleviation of metal poisoning fraction

Signs of renal dysfunction such as proteinuria appear earlier and at a lower dose on chronic administration of Cd in MT-null mice than in wild-type mice (Fig. 1). These results clearly suggest that constant expression of MT mitigates the toxicity of Cd (Liu et al. 1998). Liver disorders as signs of chronic poisoning by repeated administration of Cd appear more markedly in MT-null mice than in wild mice (Habeebu et al. 2000a). In these mice with liver disorders, apoptosis instead of necrosis was observed. The mechanism of induction of apoptosis by Cd is a problem that urgently requires a solution. Two mechanisms of the occurrence of kidney disorders have been suggested: (1) The uptake of Cd-MT derived from the liver by kidney cells and (2) an increase in active Cd due to excessive degradation of Cd-MT that accumulated in the kidney. The occurrence of direct poisoning by Cd not mediated by Cd-MT derived from the liver is considered to be possible. Moreover, decreases in bone mineral density and bone Ca concentration and an increase in osteoid were observed notably in MT-null mice repeatedly administered Cd (Habeebu et al. 2000b). No difference was observed in bone Cd concentration between

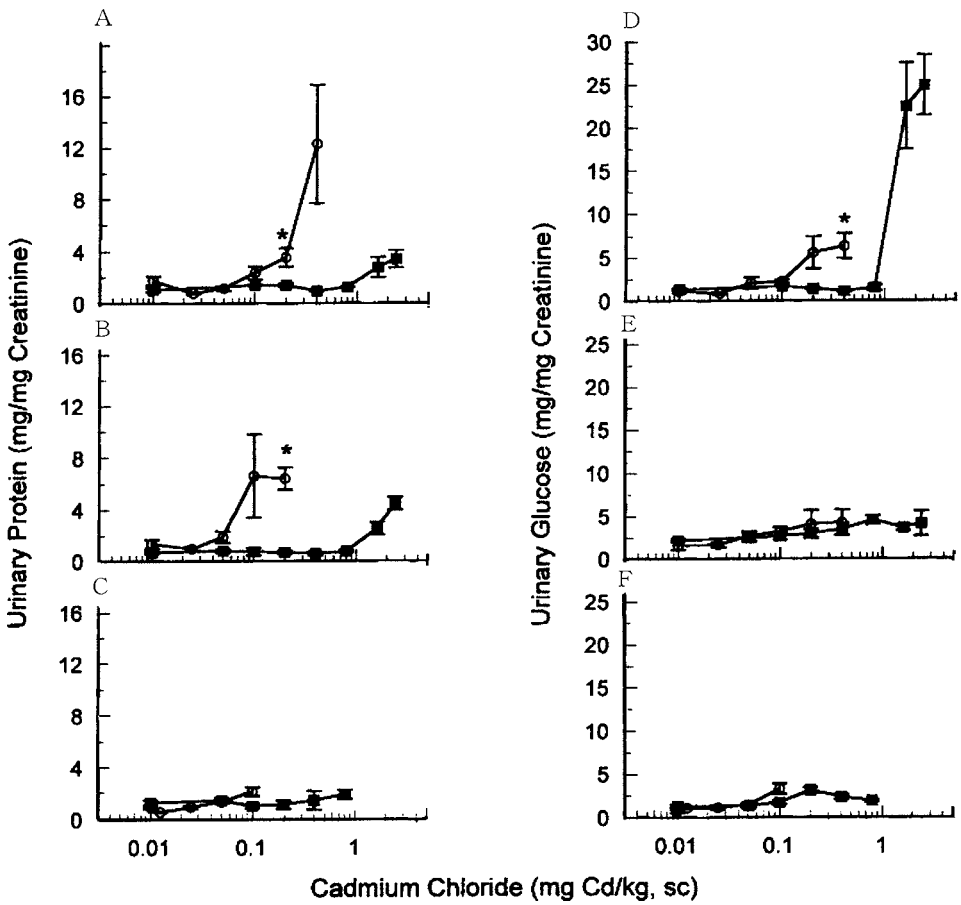

Fig. 1. Urinary excretion of protein and glucose following chronic $CdCl_2$ administration to control (0.05 to 2.4 mg/kg, seconds) and MT-null mice (0.0125 to 0.4 mgCd/kg, seconds) daily for 3 (A, D), 6 (B, E), and 10 (C, F) weeks. Figures correspond to total protein (left) and glucose (right). —○—, control, —■—, MT-null mice. Data are mean±S.E. of 6 to 8 mice.
*Significantly different from controls ($p<0.05$) (Liu et al. 1998).
■, Control; □, MT-null.

MT-null mice and wild-type mice. These results established that Cd induces osteoporosis and osteomalacia and that MT prevents these bone disorders.

The lethal toxicity of arsenicals (As), which does not bind with MT, appears at a lower dose in MT-null mice than in wild mice, indicating that MT mitigates the toxicity of As (Liu et al. 2000). Also, methylmercury has been considered not to be involved in reactions mediated by MT. The cytotoxicity of methylmercury is greater in astrocytes derived from MT-null mice than that from wild-type mice, suggesting that MT is involved in the mitigation of the toxicity of methylmercury (Yao et al. 2000). Further detailed evaluation is needed.

Concerning the relationships between the accumulated doses of heavy metals and the mortality, Cd was seven times more likely to develop toxicity in MT-null mice than in wild mice (Table 1). However, this ratio was unexpectedly low at 1.3–1.4 for Cu and Hg (Park et al. 2001). Why the protective effects of MT against Cu and Hg, which have greater affinity for thionein than Cd, are weaker than that against Cd must also be evaluated. Clarification of whether this difference is due to the difference in the metabolism of respective metals or the difference in the action mechanism is an important step for clarification of the

TABLE 1. *Summary of LD_{50} in wild-type and MT-null mice after repeated subcutaneous administration of Cd and other metals*[a]

Metal	Unit	LD_{50}		Potency ratio
		Wild-type mice	MT-null mice	
Cd	mg kg^{-1}	33.6 (29.4–39.7)	4.9 (4.1–5.8)	6.9 (2.7–41.4)[b]
Zn	mg kg^{-1}	1.9 (1.6–2.3)	0.8 (0.6–0.9)	2.4 (1.5–8.1)[b]
Cu	mg kg^{-1}	169 (147–196)	123 (105–142)	1.4 (1.1–1.9)[b]
As	mg kg^{-1}	83 (71–100)	58 (50–69)	1.4 (1.1–2.3)[b]
Hg	mg kg^{-1}	225 (186–285)	172 (142–217)	1.3 (1.0–1.9)
Pb	mg kg^{-1}	16.9 (13.7–22.3)	17.2 (13.6–23.0)	1.0 (0.7–1.4)
Fe	mg kg^{-1}	10.8 (9.0–13.0)	11.4 (9.7–13.5)	0.9 (0.7–1.2)

[a]Values in parentheses indicate 95% confidence interval. Statistical analysis, including determination of potency ratio and statistical significance, was carried out by Probit analysis and Fieller's theorem.
[b]The LD_{50} of wild-type mice is significantly higher than that of MT-null mice, as determined by the non-overlap of the 95% confidence intervals of wild-type and MT-null mice' LD_{50}s (Probit analysis), as well as I is not contained within the 95% confidence interval of the potency ratio (Fieller's theorem).

toxicity of heavy metals and prevention of poisoning by them.

Possibility of the occurrence of Cd poisoning due to environmental contamination and MT

Jarup et al. (1998) tentatively calculated the possibility of the occurrence of kidney disorders in 7% of the population due to environmental contamination by Cd (Fig. 2). Since some Japanese have been suggested to have a poor ability to synthesize MT (Yoshida et al. 1998), clarification of the state of latent abnormalities of kidney functions in people chronically exposed to low levels of Cd and analyses with consideration of the effects of Cd exposure in individuals with compromised kidney functions such as those with iron deficiency and the elderly are necessary.

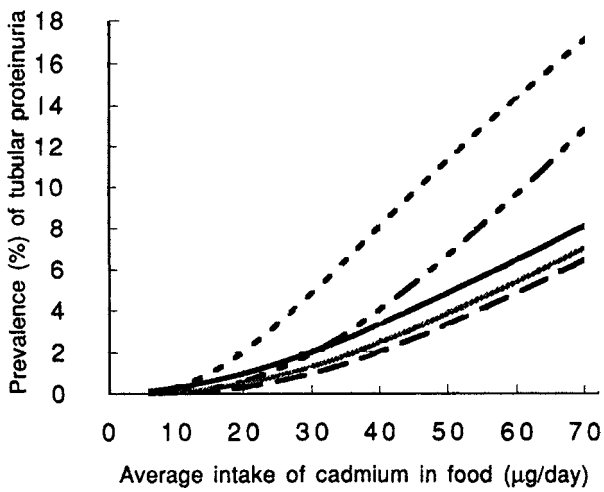

Fig. 2. Relationship between the population average cadmium (Cd) intake from food and prevalence of persons with cadmium-induced tubular damage in that population (Jarup et al. 1998).
- - , women with empty iron stores; — —, high risk group; - - —, smokers; ——, general population; ——, non smokers.

Epigenetics of MT

With the rapid advances in the genome project, the entire base sequence of the genome has been determined in some species, and the complete clarification of the genomic information, which is the plan of life, isquiching approaching. In these circumstances, epigenetics, which is the mechanism of selective activation or inactivation of gene function, has begun to attract attention. Gene expression is controlled precisely, and genes are expressed when necessary, at necessary sites, and in neces-

sary quantities. A higher mechanism for utilizing genomic information including methylation of DNA, acetylation of histone, and regulation of transcription (epigenetics) is known to be involved in this control, and clarification of the epigenetics is indispensable for understanding biological activities. Although MT is induced by a wide variety of stressors ranging from chemical to physical stressors, many of its physiological roles are still difficult to definitively explain. Transcription factors involved in the expression of MT gene induced by these stressors have already been identified, and the understanding of the epigenetics of MT will serve as a landmark in the clarification of the diverse physiological functions of MT. In this review, therefore, the epigenetics of MT is discussed briefly.

The promoter region glucocorticoid response elements (GREs), metal response elements (MREs), the antioxidant response element (ARE), and the elements activated by STAT (signal transducers and activators of transcription) are present in the upstream of the chromosome that codes for MT gene, and are known to be involved in the expression of MT induced by glucocorticoids, Zn, activated oxygen, and cytokines (Davis and Cousins 2000) (Fig. 3). Among these mechanisms of transcription activation, the mechanism of MT induction via MRE has been studied most. The MRE promoter is considered to be involved in the MT induction by metals, the MT induction by Zn that is regulated by MRE-binding transcription factor-1 (MTF-1), which is a transcription factor that binds to the MRE promoter, and MTF-1 that regulates Zn-dependent MT induction as a sensor of Zn concentration in the cytoplasm (Dalton et al. 1997; Bittel et al. 1998; Andrews 2000). Moreover, since it has been recently shown that MTF-1 is essential for the expression of MT and that coordinated actions of MTF-1 and USF-1 (basic helix-loop-helix upstream stimulatory factor-1) are involved in the

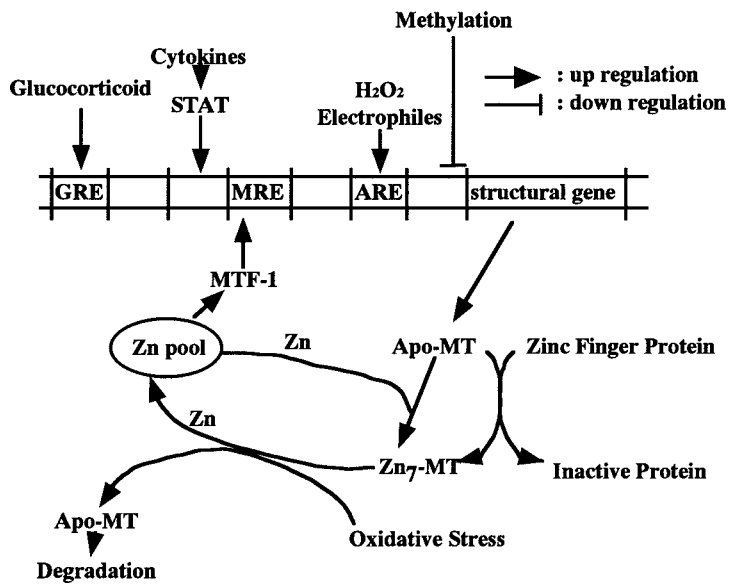

Fig. 3. Overview of metallothionein (MT) gene regulation. The MT promoter has many response elements that up-regulate transcription. These include the following: 1) metal response elements (MRE), which are activated by the metal-responsive transcription factor (MTF-1) after zinc occupancy; 2) glucocorticoid response elements (GRE); 3) elements activated by STAT (signal transducers and activators of transcription) proteins through cytokine signaling; and 4) the antioxidant (or electrophile) response elements (ARE) activated in response to redox status. Methylation may down-regulate expression in some tumor cells (Davis and Cousins 2000).

regulation of the quantity of MT expression (Andrews et al. 2001), a mechanism mediated by multiple transcription factors is suggested to be present in the mechanism of regulation of MT expression.

Although the majority of studies on the MT expression to date have dealt with the induction of gene expression, the diversity of the physiological functions of MT suggests the presence of mechanisms that suppress the MT expression. In fact, the finding that heavy metals and glucocorticoids induce MT in most organs and cell types but not in some tumor cells lines (lymphoid-derived tumor cells W7, S49) suggests the presence of a mechanism that suppresses the MT expression (Compere and Palmiter 1981). Ghoshal et al. (2000) evaluated the MT induction by Zn and Cd using rats bearing transplanted hepatoma and observed MT induction in the liver but not in the hepatoma. Since normal responses of MTF-1 were observed in the hepatoma, this finding cannot be explained by the inactivation of the transcription factor. Therefore, Ghoshal et al. (2000) examined methylation of the MT promoter regions and showed methylation of 21 CpG regions, demonstrating the presence of an MT gene silencing mechanism. Further advances in the study of epigenetics including the clarification of the silencing mechanism and the mechanisms of transcription factor regulation by the expression of MT gene, are expected to lead to the clarification of the physiological functions of MT.

MT and the brain

In the 21st century, which is called the century of the brain, the functional analysis of MT in the brain is expected to be increasingly advanced. In this article, studies on the brain and MT, particularly recent ones, are reviewed.

Zn is present in presynaptic terminals and is known to not only act as a neurotransmitter but also be released from the cell in brain ischemia and to damage the central nervous system (Koh et al. 1996). Therefore, MT, which is a Zn-binding protein, is considered to play an important role also in the brain. MT-I, MT-II, and MT-III but not MT-IV are present in the brain. Although no marked difference is observed in the quantity of expression among these isoforms, the quantity of their expression on the mRNA level differs slightly in the order of MT-I (100%)>MT-III (70%)>MT-II (50%). Also, all these isoforms are distributed over the entire brain, and their expression in the olfactory bulb, cortex, caudate nucleus, thalamus, hippocampus, brainstem, and spinal cord has been confirmed. Particularly, the quantity of MT expression, which is largest in the olfactory bulb and smallest in the eye, suggests the function of MT in sensory organs (Choudhuri et al. 1995). The quantity of MT present in astrocytes, which account for 20% of the gray matter, is about five times greater than that in neurons (Aschner 1997). Astrocytes, which are distributed near synapses, Ranvier nodes, and axons, regulate neural signals, and MT is suggested to be involved in astrocyte functions. In fact, the active oxygen production associated with the addition of tert-butyl hydroperoxide was significantly reduced in astrocytes isolated from MT transgenic mice compared with control cells (Suzuki et al. 2000). Also, the expression of MT-I and MT-II is induced in neurons and astrocytes by the addition of Zn, Cd, Hg, and dexamethasone (Kramer et al. 1996a, b). From these results, MT-I and MT-II are considered to have cell-protecting effects against various stresses in the nervous system. In contrast to the behavior of MT-I and MT-II, MT-III is not induced by these factors. MT-III is expressed specifically in the brain, and the quantity of its expression is reduced in patients with Alzheimer's disease. Therefore, changes in the expression of MT-III are considered to be related to the occurrence of degenerative nerve diseases (Uchida et al. 1991; Yu et al. 2001). In fact, a study of the quantity of MT-III expression in the brains of patients with Alzheimer's

disease using human clinical samples showed a significant decrease in the MT-III level (Yu et al. 2001).

Localization of MT in the cell

An understanding of the localization of MT in the cell is important to clarify the mechanisms of its physiological functions. MT is usually localized in the cytoplasm and is transferred into the nucleus in partial hepatectomy, suggesting cell-cycle-dependent changes in the localization of MT in the cell (Tohyama et al. 1993). In fact, MT concentration in the cytoplasm was the highest in the late G1 phase and the G1/S phase in a study using human colonic cancer cells (HT-29) (Nagel and Vallee 1995). When the cell cycle is arrested in the G1/S phase by aphidicolin treatment, MT accumulates with Zn in the nucleus. When aphidicolin is removed by washing these arrested cells, the cell cycle progresses synchronously with the S phase. With the advance of the cell cycle to the G2/M phase, MT is transferred into the cytoplasm (Apostolova and Cherian 2000). MT is also present in the cytoplasm in the G0 and G1 phases in first-generation rat hepatocytes, but it moves into the nucleus in the early S phase (Tsujikawa et al. 1991). Many reports concerning the mechanism of this transfer of MT into the nucleus have appeared recently. Nagano et al. (2000) showed that MT moves into the nucleus in a manner independent of WGA (wheat germ agglutinin) and dependent on the cytoplasmic fraction in a study using digitonin-treated BALB/c 3T3 cells. Woo et al. (2000), in contrast, suggested that MT moves into the nucleus in a manner dependent on WGA and independent of the cytoplasmic fraction in a study using digitonin-treated human SCC25 carcinoma cells. From these results, the route of transfer of MT into the nucleus is considered to vary with the cell type. Also, since the reported signal of transfer into the nucleus is not observed in MT, a factor that controls the transfer of MT into the nucleus is considered to be present. Generally, the localization of protein in the cell is determined by two mechanisms, i.e., the localization signal sequence present in the protein and the localization of mRNA in the cell. Levadoux et al. (1999) analyzed the localizations of MT mRNA and MT protein in the cell and demonstrated that the localization of MT mRNA in the cytoskeleton near the nucleus is a factor that determines the transfer of MT into the nucleus in the G1/S phase.

Woo and Lazo (1997) evaluated the relationship between the localization of MT in the cell and its physiological functions by having cytoplasm-localized MT and nucleus-localized MT expressed in MT-null cells. Cytoplasm-localized MT had a suppressive effect on oxidative damage by *tert*-butylhydroperoxide and the cytotoxicity of heavy metals, but nucleus-localized MT showed no such suppressive effect. On the other hand, nucleus-localized MT showed a cytoprotective effect against the cytotoxicity of N-methyl-N′-nitro-N-nitrosoguanidine. Nuclear MT also showed a protective effect against DNA damage caused by ultraviolet rays and hydrogen peroxide (Levadoux-Martin et al. 2001). The localization of MT in the cell was also suggested to be important in the alleviation of the cardiotoxicity of doxorubicin in a study using MT-Tg mice (Zhou and Kang 2000). A correlation was observed between the malignancy of cancer and the quantity of MT expressed in studies using tumor cells, and suppression of apoptosis associated with excess expression of MT is considered to be a cause of exacerbation of tumors (Cherian 1994; Hengstler et al. 2001). In addition, the localization of MT in the cell has been reported to be related to the exacerbation of some tumors (Jayasurya et al. 2000), therefore the quantity and localization of MT expression in the cell are considered to be important factors in the carcinogenic process. Recently, also, Ye et al. (2001) showed that MT is localized between the internal and external membranes of the mitochondria (Fig. 4) and that this

MT localized in mitochondria regulates oxygen respiration. Since mitochondria are sites of constant production of active oxygen as well as of oxygen respiration, the relationship of this localization with the reactive oxygen scavenging activity of MT is of interest. The authors reported not only the constant presence of in the mitochondria but also the possibility of the presence of MT in hepatic mitochondria in Cd-treated and Cd-MT-treated rats. Recently, Tang and Shaikh (2001) suggested that Cd poisoning is caused by the disturbance of mitochondrial respiration, and the analysis of the significance of the presence of MT in mitochondria is needed.

Radical scavenging activity

There have been a number of general discussions to date concerning the reactive oxygen scavenging reactivity of MT (Sato 1992; Sato and Bremner 1993). In this paper, recent reports of evaluations using MT-null mice and MT-Tg mice are reviewed.

MT was noted first for its function as a heavy metal detoxicating protein, but it was suggested to have the activity of an reactive oxygen scavenger (Sato 1992; Sato and Bremner 1993), and induction of MT by paraquat and cytokines with reactive oxygen producing activities was reported in normal mice (Sato 1991, 1994; Sato et al. 1995). In fact, because MT-I and MT-II genes are expressed frequently in mice defective in Cu, Zn-superoxide dismutase, which is localized in the cytoplasm, MT is considered to have an reactive oxygen scavenging activity (Ghoshal et al. 1999). Recently, the reactive oxygen scavenging activity of MT has been reevaluated using MT-null mice defective

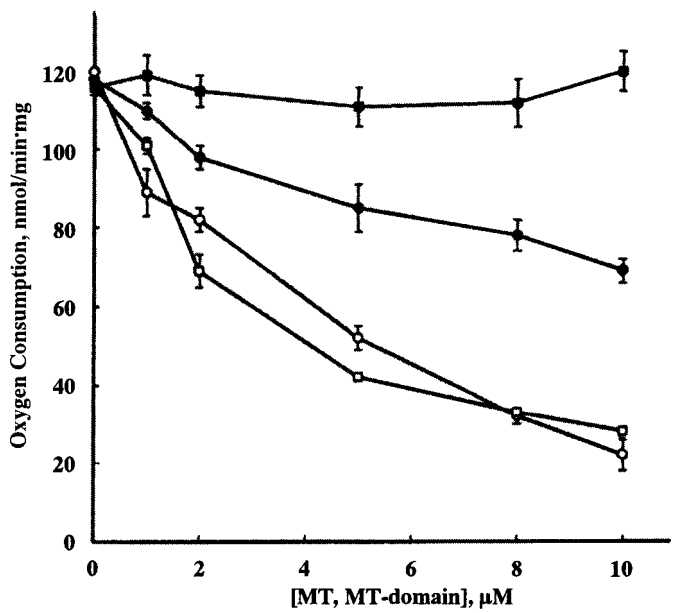

Fig. 4. Inhibition of state 3 mitochondrial respiration by MT isoforms and zinc-reconstituted MT-domain peptides. Mitochondria corresponding to 500 μg of protein were injected into the electrochemical cell (450 μl) and incubated for 3 minutes. Succinate and ADP were then added to final concentrations of 3 mM and 0.5 mM, respectively. MT (up to 15 μl) was added when about half of the ADP was consumed (after about 1 minute). The data for rabbit Zn7-MT-I and Zn7-MT-II were from two preparations of mitochondria. Incubation buffer (2–15 μl) or BSA (15 μg in 10 μl of buffer) had no effect. Zn-reconstituted a-domain and b-domain of human MT-II were also equilibrated with incubation buffer. Addition of 10 μl of buffer served as a control. Standard errors are based on at least two measurements (Ye et al. 2001).
■, human MT-II a-domain; ●, rabbit MT-I; ○, rabbit MT-II; □, human MT-II b-domain.

in MT-I and MT-II genes and MT-Tg mice excessively expressing MT-I and MT-II genes. Lazo et al. (1995) evaluated the sensitivity of fibroblasts isolated from MT-null mice to reactive oxygen and demonstrated that MT has a scavenging activity of reactive oxygen species on the cellullar level. Satoh et al. (1988, 2000) evaluated the effects of MT on the renal toxicity of cisplatin using MT-null mice and showed that MT significantly suppresses the nephrotoxicity of cisplatin. Also, concerning evaluations using doxorubicin, Kimura et al. (2000) showed that MT mitigates the cardiotoxicitiy of doxorubicin both in vivo and in vitro using MT-Tg mice and myocardial cells isolated from MT-Tg mice. The group of Kang et al. (2000) conducted systematic evaluations using MT-Tg mice that specifically express MT in the heart and showed that MT suppresses the doxorubicin-induced lipid peroxide production and that MT significantly suppresses the induction of apoptosis of myocardial cells due to the cardiotoxicity of doxorubicin (Wu and Kang 1998, 1999). They further noted activation of p38 mitogen-activated protein kinase (p38 MAP kinase) during induction of myocardial cell apoptosis by doxorubicin and clarified that suppression activation of p38 MAP kinase is involved in the suppression of apoptosis by MT (Kang et al. 2000). These results also suggest that MT regulates intracellular signal transmission via the regulation of reactive oxygen species. Furthermore, it was recently reported in succession that MT regulates the activation of nuclear factor-kB by tumor necrosis factor-α and nitric oxide signal transmission (Sakurai et al. 1999; Pearce et al. 2000). Since reactive oxygen acts as a messenger molecule of intracellular signals, the reactive oxygen scavenging activity of MT may be closely related to the regulation of intracellular signal transmission. The future development of this research is anticipated.

Summary

Since MT is induced equally by chemical, physical, and psychological stresses as long as the stress has a negative effect on the body, it has attracted attention from a toxicological viewpoint. Recently, however, the weight of research is shifting to the clarification of the physiological functions of MT, and knowledge about the Zn-retaining activity of MT, the mechanism of the regulation of MT expression, and the intracellular localization of MT is steadily increasing. Particularly, buds for new developments of studies on MT are observed in the reports concerning the presence of the mechanism of suppression of MT gene expression by methylation (Ghoshal et al. 2000) and the localization of MT in the mitochondria (Ye et al. 2001).

Although MT was shown to mitigate the toxicity of heavy metals such as Cd and Hg in the routine level of its expression in experiments using MT-null mice, clarification of the mechanism of this mitigation is still necessary. Moreover, the presence of a substitute mechanism for the physiological functions of MT suggested through the preparation of MT-null mice demands systematic and exhaustive analyses in MT research. MT is a stress protein that markedly responds to external stimuli, and the clarification of its physiological significance is awaited. The elucidation of the physiological function of MT is expected to progress rapidly and boldly by means of both experimental confirmation based on a single principle and systematic and exhaustive analysis utilizing genome science, transcritome science, and proteome science.

References

Andrews, G.K. (1990) Regulation of metallothionein gene expression. *Prog. Food Nutr. Sci.*, **14**, 193–258.

Andrews, G.K. & Geiser, J. (1999) Expression of the mouse metallothionein-I and -II genes pro-

vides a reproductive advantage during maternal dietary zinc deficiency. *J Nutr.*, **129**, 1643–1648.

Andrews, G.K. (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem. Pharmacol.*, **59**, 95–104.

Andrews G.K., Lee, D.K., Ravindra, R., Lichtlen, P., Sirito, M., Sawadogo, M. & Schaffner, W. (2001) The transcription factors MTF-1 and USF1 cooperate to regulate mouse metallothionein-I expression in response to the essential metal zinc in visceral endoderm cells during early development. *EMBO J.*, **20**, 1114–1122.

Apostolova, M.D. & Cherian, M.G. (2000) Delay of M-phase onset by aphidicolin can retain the nuclear localization of zinc and metallothionein in 3T3-L1 fibroblasts. *J. Cell. Physiol.*, **183**, 247–253.

Aschner, M. (1997) Astrocyte metallothioneins (MTs) and their neuroprotective role. *Ann. NY. Acad. Sci.*, **825**, 334–347.

Bittel, D., Dalton, T.D., Samson, S., Geclamu, L. & Andrews, G.K. (1998) The DNA-binding activity of metal response element-binding transcription factor-1 is activated in vivo and in vitro by zinc, but not by other transition metals. *J. Biol. Chem.*, **273**, 7127–7133.

Cherian, M.G. (1994) The significance of the nuclear and cytoplasmic localization of metallothionein in human liver and tumor cells. *Environ. Health Perspect.*, **102** (Suppl. 3), 131–135.

Choudhuri, S., Kramer, K.K., Berman, N.E.J., Dalton, T.P., Andrews, G.K. & Klaassen, C.D. (1995) Constitutive expression of metallothionein genes in mouse brain. *Toxicol. Appl. Pharmacol.*, **131**, 144–154.

Compere, S.J. & Palmiter, R.D. (1981) DNA methylation controls the inducibility of the mouse metallothionein-I gene lymphoid cells. *Cell*, **25**, 233–240.

Dalton, T.P., Fu, K., Palmiter, R.D. & Andrews, G.K. (1996) Transgenic mice that overexpress metallothionein-I resist dietary zinc deficiency. *J. Nutr.*, **26**, 825–833.

Dalton, T.P., Bittel, D. & Andrews, G.K. (1997) Reversible activation of the mouse metal response element-binding transcription factor-1 DNA binding involves zinc interactions with the zinc-finger domain. *Mol. Cell. Biol.*, **17**, 2781–2789.

Davis, S.R. & Cousins, R.J. (2000) Metallothionein expression in animals: A physiological perspective on function. *J. Nutr.*, **130**, 1085–1088.

Ghoshal, K., Majumder, S., Li, Z., Bray, T.M. & Jacob, S.T. (1999) Transcriptional induction of metallothionein-I and -II genes in the livers of Cu, Zn-superoxide dismutase knockout mice. *Biochem. Biophys. Res. Commun.*, **264**, 35–742.

Ghoshal, K., Majumder, S., Li, Z., Dong, X. & Jacob, S.T. (2000) Suppression of metallothionein gene expression in a rat hepatoma because of promoter-specific DNA methylation. *J. Biol. Chem.*, **275**, 539–547.

Habeebu, S.S., Liu, J., Yaping, J.L. & Klaassen, C.D. (2000a) Metallothionein-null mice are more sensitive than wild-type mice to liver injury induced by repeated exposure to cadmium. *Toxicol. Sci.*, **55**, 223–232.

Habeebu, S.S., Liu, J., Yaping, J. L. & Klaassen, C.D. (2000b) Metallothionein-null mice are more sensitive than wild-type mice to chronic CdCl2-induced bone injury. *Toxicol. Sci.*, **56**, 211–219.

Hengstler, J.G., Pilch, H., Schmidt, M., Dahlenburg, H., Sagemuller, J., Schiffer, I., Oesch, F., Knapstein, P.G., Kaina, B. & Tanner, B. (2001) Metallothionein expression in ovarian cancer in relation to histopathological parameters and molecular markers of prognosis. *95 Int. J. Cancer.*, **95**, 121–127.

Jarup, L., Berglund, M., Elinder, C.G., Nordberg, O. & Vahter, M. (1998) Health effects of cadmium exposure - a review of the literature and a risk estimate. *Scand. J. Work Environ. Health*, **24**, Suppl., 1–52.

Jayasurya, A., Bay, B.H., Yap, W.M. & Tan, N.G. (2000) Correlation of metallothionein expression with apoptosis in nasopharyngeal carcinoma. *Br. J. Cancer*, **82**, 1198–1203.

Kagi, J.H.R. & Vallee, B.L. (1960) Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J. Biol. Chem.*, **235**, 3460–3465.

Kang, Y.J., Zhou, Z.X., Wang, G.W., Buridi, A. & Klein, J.B. (2000) Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. *J. Biol. Chem.*, **275**, 13690–13698.

Kelly, E.J., Quaife, C.J., Froelick, G.J. & Palmiter, R. D. (1996) Metallothionein I and II protect

against zinc deficiency and zinc toxicity in mice. *J. Nutr.*, **126**, 1782–1790.

Kimura, T., Fujita, I., Itoh, N., Muto, N., Nakanishi, T., Takahashi, K., Azuma, J. & Tanaka, K. (2000) Metallothionein acts as a cytoprotectant against doxorubicin toxicity. *J. Pharm. Exp. Ther.*, **292**, 299–302.

Koh, J.Y., Suh, S.W., Gwag, B.J., He, Y.Y., Hsu, C.Y. & Choi, D.W. (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science*, **272**, 1013–1016.

Kondoh, M. & Sato, M. (2002) Protective stress protein system: Metallothionein. *Jpn. J. Hyg.*, **56**, 634–640.

Kramer, K.K., Liu, J., Choudhuri, S. & Klaassen, C.D. (1996a) Induction of metallothionein mRNA and protein in murine astrocyte cultures. *Toxicol. Appl. Pharmacol.*, **136**, 94–100.

Kramer, K.K., Zoelle, J.T. & Klaassen, C.D. (1996b) Induction of metallothionein mRNA and protein in primary murine neuron cultures. *Toxicol. Appl. Pharmacol.*, **141**, 1–7.

Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E., Choo, K.H.A. & Pitt, B.R. (1995) Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *J. Biol. Chem.*, **270**, 5506–5510.

Levadoux, M., Mahon, C., Beattie, J.H., Wallace, H.M. & Hesketh, J.E. (1999) Nuclear import of metallothionein requires its mRNA to be associated with the perinuclear cytoskeleton. *J. Biol. Chem.*, **274**, 34961–34966.

Levadoux-Martin, M., Hesketh, J.E., Beattie, J.H. & Wallace, H.M. (2001) Influence of metallothionein-1 localization on its function. *Biochem. J.*, **355**, 473–479.

Liu, J., Liu, Y., Michalska, A.E., Choo, K.H.A. & Klaassen, C.D. (1996) Distribution and retention of cadmium in metallothionein I and II null mice. *Toxicol. Appl. Pharmacol.*, **136**, 260–268.

Liu, J., Yaping, J.L., Habeebu, S.S.M. & Klaassen, C.D. (1998) Susceptibility of MT-null mice to chronic $CdCl_2$-Induced nephrotoxicity indicates that renal injury is not mediated by the CdMT complex. *Toxicol. Sci.*, **46**, 197–203.

Liu, J., Liu, Y., Goyer, R.A., Achanzar, W. & Waalkes, M.P. (2000) Metallothionein-I/II null mice are more sensitive than wild-type mice to the hepatotoxic and nephrotoxic effects of chronic oral or injected inorganic arsenicals. *Toxicol. Sci.*, **55**, 460–467.

Margoshes, M. & Vallee, B.L. (1957) A cadmium protein from equine kidney cortex. *J. Am. Chem. Soc.*, **79**, 4813–4814.

Masters, B.A., Kelly, E.J., Quaife, C.J., Brinster, R.L. & Palmiter, R.D. (1994) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc. Natl. Acad. Sci. USA*, **91**, 584–588.

Michalska, A.E. & Choo, K.H.A. (1993) Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc. Natl. Acad. Sci. USA*, **90**, 8088–8092.

Nagano, T., Itoh, N., Ebisutani, C., Takatani, T., Miyoshi, T., Nakanishi, T. & Tanaka, K. (2000) The transport mechanism of metallothionein is different from that of classical NLS-bearing protein. *J. Cell Physiol.*, **185**, 440–446.

Nagel, W.W. & Vallee, B.L. (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. *Proc. Natl. Acad. Sci. USA*, **92**, 579–583.

Palmiter, R.D. (1995) Constitutive expression of metallothionein-III (MT-III), but not MT-I, inhibits growth when cells become zinc deficient. *Toxicol. Appl. Pharmacol.*, **135**, 139–146.

Park, J.D., Liu, Y., Klaassen, C.D. (2001) Protective effect of metallothionein against the toxicity of cadmium and other metals (1). *Toxicology.*, **163**, 93–100.

Pearce, L.L., Gandley, R.E, Han, W., Wasserloos, K., Stitt, M., Kanai, A.J., McLaughlin, M.K., Pitt, B.R. & Levitan, E.S. (2000) Role of metallothionein in nitric oxide signaling as revealed by a green fluorescent fusion protein. *Proc. Natl. Acad. Sci. USA*, **97**, 477–482.

Philcox, J.C., Coyle, P., Michalska, A., Choo, K.H.A. & Rofe, A.M. (1995) Endotoxin-induced inflammation does not cause hepatic zinc accumulation in mice lacking metallothionein gene expression. *Biochem. J.*, **308**, 543–546.

Philcox, J.C., Sturkenboom, M., Coyle, P. & Rofe, A.M. (2000) Metallothionein in mice reduces intestinal zinc loss during acute endotoxin inflammation, but not during starvation or dietary zinc restriction. *J. Nutr.*, **130**, 1901–1909.

Quaife, C.J., Findley, S.D., Erickson, J.C., Froelick, G. J., Kelly, E.J., Zambrowicz, B.P. & Palmiter, R.D. (1994) Inhibition of a new metallothionein isoform (MT-IV) occurs during differ-

entiation of stratified squamous epithelia. *Biochemistry*, **33**, 7250–7259.

Quaife, C.J., Kelly, E.J., Masters, B.A., Brinster, R.L. & Palmiter, R.D. (1998) Ectopic expression of metallothionein-III causes pancreatic acinar cell necrosis in transgenic mice. *Toxicol. Appl. Phamacol.*, **148**, 148–157.

Sakurai, A., Hara S., Okano, N., Kondo, Y., Inoue, J. & Imura, N. (1999) Regulatory role of metallothionein in NF-*k*B activation. *FEBS Lett.*, **455**, 55–58.

Satoh, M., Naganuma, A. & Imura, N. (1988) Metallothionein induction prevents toxic side effects of cisplatin and adriamycin used in combination. *Cancer Chemother. Pharmacol.*, **21**, 176–178.

Sato, M. (1991) Dose-dependent increases in metallothionein synthesis in the lung and liver of paraquat-treated rats. *Toxicol. Appl. Pharmacol.*, **107**, 98–105.

Sato, M. (1992) Biological antioxidant defense system and metallothionein. *Jpn. J. Toxicol. Environ. Health*, **38**, 228–239.

Sato, M. & Bremner, I. (1993) Oxygen free radicals and metallothionein. *Free Rad. Biol. Med.*, **14**, 325–337.

Sato, M. (1994) A protein as defense system: metallothionein In: *Toxicology Today* edited by H. Sato, Kimpodoh, Kyoto, Japan, pp. 259–271.

Sato, M., Sasaki, M. & Hojo, H. (1994) Differential induction of metallothionein synthesis by interleukin-6 and tumor necrosis factor-a in rat tissues. *Int. J. Immunopharmacol.*, **16**, 187–195.

Sato, M., Sasaki, M. & Hojo, H. (1995) Antioxidative roles of metallothionein and manganese superoxide dismutase induced by tumor necrosis factor-a and interleukin-6. *Arch. Biochem. Biophys.*, **316**, 738–744.

Satoh, M., Shimada, A., Zhang, B. & Tohyama, C. (2000) Renal toxicity caused by cisplatinum in glutathione-depleted metallothionein-null mice. *Biochem. Pharmacol.*, **60**, 1729–1734.

Suzuki, Y., Apostolova, M.D. & Cherian, M.G. (2000) Astrocyte cultures from transgenic mice to study the role of metallothionein in cytotoxicity of tert-butyl hydroperoxide. *Toxicology*, **145**, 51–62.

Tang, W. & Shaikh, Z.A. (2001) Renal cortical mitochondrial dysfunction upon cadmium metallothionein administration to Sprague-Dawley rats. *J. Toxicol. Environ. Health A.*, **63**, 221–235.

Tohyama, C., Suzuki, J.S., Hemelraad, J., Nishimura, N. & Nishimura, H. (1993) Induction of metallothionein and its localization in the nucleus of rat hepatocytes after partial hepatectomy. *Hepatology*, **18**, 1193–1201.

Tsujikawa, K., Imai, T., Kakutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R. & Shimizu, N. (1991) Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. *FEBS Lett.*, **283**, 239–242.

Uchida, Y., Takio, K., Titani, K., Ihara, Y. & Tomonaga, M. (1991) The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron*, **7**, 337–347.

Wang, G.W. & Kang, Y.J. (1999) Inhibition doxorubicin toxicity in cultured neonatal mouse cardiomyocytes with elevated metallothionein levels. *J. Pharm. Exp. Ther.*, **288**, 938–944.

Woo, E.S. & Lazo, J.S. (1997) Nucleocytoplasmic functionality of metallothionein. *Cancer Res.*, **57**, 4236–4241.

Woo, E.S., Dellapiazza, D., Wang, A. & Lazo, J. (2000) Energy-dependent nuclear binding dictates metallothionein localization. *J. Cell Physiol.*, **182**, 69–76.

Wu, H.Y. & Kang, Y.J. (1998) Inhibition of buthionine sulfoximine-enhanced doxorubicin toxicity in metallothionein overexpressing transgenic mouse heart. *J. Pharm. Exp. Ther.*, **287**, 515–520.

Yao, C.P., Allenn, J.W., Mutkus, L.A., Xu, S.B. & Tan, K.H. (2000) Foreign metallothionein-I expression by transient transfection In MT-I and MT-II astrocytes confers increased protection against acute methylmercury cytotoxicity. *Brain Res.*, **855**, 32–38.

Ye, B., Maret, W. & Vallee, B.L. (2001) Zinc metallothionein imported into liver mitochondria modulates respiration. *Proc. Natl. Acad. Sci. USA*, **98**, 2317–2322.

Yoshida, M., Ohta, H., Yamauchi, Y., Seko, Y., Sagi, M., Yamazaki, K. & Sumi, Y. (1998) Age-dependent changes in metallothionein levels in liver and kidney of the Japanese. *Biological Trace Elem. Res.*, **63**, 167–175.

Yu, W.H., Lukiw, W.J., Bergeron, C., Niznik, H.B. & Fraser, P.E. (2001) Metallothionein III is reduced in Alzheimer's disease. *Brain Res.*,

894, 37–45.

Zhou, Z. & Kang, J.Y. (2000) Immunocytochemical localization of metallothionein and its relation to doxorubicin toxicity in transgenic mouse heart. *Am. J. Pathol.*, **156**, 1653–1662.