The Antioxidant Function of Metallothionein in the Heart (44451)

Y. JAMES KANG[1]

Departments of Medicine, Pharmacology and Toxicology, University of Louisville School of Medicine, and The Jewish Hospital Heart and Lung Institute, Louisville, Kentucky 40292

Abstract. **The antioxidant function of metallothionein (MT) was first suggested in the early 1980s. Studies *in vitro* have revealed that MT reacts directly with reactive oxygen species, including superoxide and hydroxyl radicals and hydrogen peroxide. These reactions have never been demonstrated in intact animal studies. Nevertheless, both pharmacologic and genetic studies have shown that MT functions in protection against oxidative injury *in vivo*. In particular, the antioxidant function of MT in the heart has been explored extensively. The data gathered from recent studies using a cardiac-specific, MT-overexpressing transgenic mouse model have provided direct evidence to support this physiological role of MT. Under acute and chronic oxidative stress conditions such as treatment with doxorubicin, ischemia-reperfusion, and dietary copper restriction, MT-overexpressing transgenic mouse hearts displayed a marked resistance to the injurious consequences, including biochemical, pathological, and functional alterations. This protective action of MT correlates with its inhibition of reactive oxygen species–induced lipid peroxidation. A critical elucidation of the mechanism of action of MT as an antioxidant *in vivo* remains to be achieved. However, the combination of recent understanding of the zinc cluster structure of MT and novel molecular genetic approaches has provided the basis for further advancement in this field.** [P.S.E.B.M. 1999, Vol 222]

Metallothionein (MT) is a transition metal–binding protein that has been studied for more than 40 years since its discovery in 1957 (1). MT is found in all eukaryotes as well as some prokaryotes (2). A comprehensive understanding of the physical, chemical, and biochemical properties of MT has been achieved (3); however, a critical evaluation of the biological functions of MT has remained unusually challenging. Although MT is implicated in a diversity of intracellular functions (4), the only consensus among MT researchers for its biological function thus far is its role in detoxification of heavy metals (5, 6). This has been demonstrated in both single-cell eukaryotes and mammals in relation to cytotoxicities of copper and cadmium. In yeast, MT typically binds copper (7, 8). Overexpression of MT confers resistance to copper toxicity, and the lack of MT in mutants sensitizes the cells to copper toxicity (9, 10). In mammals, MT binds predominantly zinc (11). However, under the conditions of copper or cadmium overload, zinc can be readily displaced by these metals (12). Many studies have demonstrated that the sensitivity of cultured mammalian cells to cadmium is related to the amount of MT expression (13, 14). Cells that contain an excess amount of MT are resistant to cadmium toxicity (15), whereas cell lines that cannot synthesize any MT are sensitive to cadmium (16). Transgenic mice that overexpress MT are also resistant to cadmium toxicity (17); conversely, MT-I and -II knock-out mice are sensitive (18, 19). These observations agree with the original discovery of cadmium-MT in horse kidney (1) and fit well with genetic studies indicating that MT genes are regulated by metals (20, 21). However, the line of reasoning that MT's primary role is detoxification of transition metals does not follow the rule of evolutionary conservation. Because the structure of MT

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[1] To whom requests for reprints should be addressed at Department of Medicine, University of Louisville School of Medicine, 530 S. Jackson St., 3rd Floor, Ambulatory Care Building, Louisville, KY 40292. E-mail: yjkang01@athena.louisville.edu

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is highly conserved, it likely performs an evolutionarily conserved function, rather than a function that solves the cell's problem with relatively recent pollution (22, 23).

The biological function of MT is likely related to the physiologically relevant metals that this protein binds. In mammals, MT is found to bind zinc and copper under normal physiological conditions. Both zinc and copper are trace metals that are essential for life. Recent studies have produced strong evidence to support the idea that MT functions as a metal chaperone for the regulation of gene expression and for synthesis and functional activity of proteins, such as metalloproteins and metal-dependent transcription factors (24–28). MT could thus serve as a reservoir of essential metals such as zinc and copper. This might dictate dual functions of metal-MTs: 1) preventing metal toxicity under overload conditions; and 2) donating the metals to apo-metalloproteins under physiological conditions. Studies *in vitro* indicate that the transfer of metals from MT to an acceptor is possible (29, 30), and glutathione (GSH) has been shown to facilitate such a transfer (31, 32). Most of these metal transfer studies have focused on MT regulation of zinc homeostasis (29–34). Zinc is much less toxic to living systems than other transition metals, such as copper and cadmium, and zinc participates in protein, nucleic acid, carbohydrate, and lipid metabolism, as well as the regulation of gene expression, and cell growth and differentiation (35, 36). MT has been found not only in the cytoplasm but also in the nucleus (37). This is interesting because under physiological conditions, MT predominantly binds zinc, which is involved in control of gene expression and cell proliferation. The possible significance of its nuclear localization has been studied in 3T3-L1 fibroblasts. MT was localized in the nucleus at late G1/early S-phase after cell growth was blocked with Aphidicolin, an inhibitor of DNA polymerase-α. Slowing the progression of the cell cycle with Aphidicolin led to relocalization of MT to the cytoplasm at the end of M-phase (38). Further studies suggested that the nuclear translocation of MT is dependent on MAP-kinase and PI3-kinase activities (38), and that MT in the nucleus can regulate the activity of nuclear factor-κB (NFκB) (39). It is foreseeable that more biological functions related to MT as a zinc chaperone will be revealed.

Other suggested functions of MT remain controversial. An idea derived mainly from *in vitro* experimental data is that MT functions as a free radical scavenger (40–42). Although the *in vivo* antioxidant function of MT is still subject to debate, recent studies using state-of-the-art experimental approaches have produced evidence that MT functions in protection against oxidative injury *in vivo*. For example, MT gene expression is upregulated under virtually all prooxidant conditions thus far examined (43–45). MT has also been shown to protect against toxicity of reactive oxygen and nitrogen species in multiple organ systems in mammals. The following will discuss the experimental data from both *in vitro* and *in vivo* studies in search of antioxidant functions of MT.

MT as an Antioxidant

The hypothesis that MT functions as an antioxidant against reactive oxygen and nitrogen species has received extensive experimental support from *in vitro* studies. Studies using a cell-free system have demonstrated the ability of MT as a free radical scavenger (40–42). However, these results have not been demonstrated in intact animal studies, and most *in vivo* experimental data provide only indirect evidence for the free radical scavenging action of MT. Nevertheless, many *in vivo* studies indicate that MT indeed provides protection against oxidative injury in multiple organ systems, strongly implicating its antioxidant function.

That MT functions as an antioxidant was first suggested in a study examining the effect of MT on radiosensitivity of cultured human epithelial (HE) cell line and mouse fibroblast (C1 1D) cells (46). Following this study, a detailed examination of the kinetics and mechanism of reaction of MT with superoxide and hydroxyl radicals was undertaken (40). MT containing zinc and/or cadmium was shown to scavenge for hydroxyl and superoxide radicals produced by the xanthine/xanthine oxidase reaction system (40). The data suggested that all 20 cysteine sulfur atoms are involved in the radical quenching process, and MT appears to be an extraordinarily efficient hydroxyl radical scavenger (40). The rate constant for the reaction of hydroxyl radical with MT ($K_{\cdot OH/MT}$ = 2700) is about 340-fold higher than that with GSH ($K_{\cdot OH/GSH}$ = 8) (40). Further studies have shown that MT is about 800-fold more potent than GSH (on a molar basis) in preventing hydroxyl radical–generated DNA degradation *in vitro* (41).

Studies using cultured cells have provided further evidence to support the antioxidant function of MT. Erythrocyte ghosts incubated with xanthine/xanthine oxidase/Fe(III) underwent hydrogen peroxide- and superoxide-dependent lipid peroxidation (42). Both cadmium- and zinc-MT strongly inhibited lipid peroxidation when present during incubation (42). A recent study using HL-60 cells demonstrated a direct reaction of hydrogen peroxide with the sulfhydryl groups of MT (47). Moreover, this study showed that the thiolate groups in MT were the preferred targets attacked by hydrogen peroxide compared to sulfhydryl residues from GSH and other protein fractions (47). In another study, transfection of NIH 3T3 cells with a plasmid containing the mouse *MT-1* gene yielded a 4-fold increase in intracellular MT, which was localized in the cytoplasm as determined by immunofluorescence and confocal microscopy (48). These cells were six times more resistant to the cytotoxic effects of *tert*-butyl hydroperoxide, whose toxicity is mediated by free radicals, relative to control transfectants (with an inverted *MT* gene that resulted in no increase in cellular MT concentrations) (48). These MT-overexpressing NIH 3T3 cells were also 4-fold more resistant to

the cytotoxicity of the NO donor S-nitroso-N-acetylpenicillamine (49). Taken together, studies using cultured cells have demonstrated that MT protects cells from all of the toxicologically significant reactive oxygen and nitrogen species, including superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide.

Other studies were undertaken to determine whether or not MT protects against oxidative injury in intact animals. Prior to the use of genetically engineered mice, the bulk of these studies involved various MT inducers. Although these inducer studies have been informative, it should be noted that all of the agents used to induce MT were all pleiotropic, causing a panoply of biological responses. Induction of MT in rats by cadmium, zinc, α-hederin or lipopolysaccharide has been shown to increase hepatic resistance to oxidative stress (50). Pretreatment of rat with tumor necrosis factor or interleukin-6 induced MT synthesis and prevented liver damage and lipid peroxidation caused by carbon tetrachloride (45). Zinc pretreatment significantly increased MT concentrations in the renal cortex and depressed proximal tubule necrosis and acute renal failure caused by injection of gentamicin (51), whose nephrotoxicity has been shown to be mediated by hydroxyl radical. The protection conferred by MT was presumably mediated by scavenging hydroxyl radicals (52).

Rats exposed to cadmium aerosol displayed an increase in MT content in alveolar epithelial type-II cells. When isolated from these animals, the cells were found to incur less oxidant-induced cytotoxicity than controls (53). Pulmonary MT was also increased by bismuth or zinc in mice, and this MT induction correlated with suppression of carcinogenesis by *cis*-platinum or melphalan in the lung (54). Induction of MT in the lungs of mice by zinc was also found to inhibit paraquat-induced pulmonary lipid peroxidation (55). Paraquat lethality from intratracheal installation was also significantly decreased in mice pretreated with zinc; this correlated with MT levels in the lung, but not with MT contents in the liver or kidney (55). Recent studies have been extended to the function of MT in the brain (56). It was shown that MT-III, which is brain specific, is predominantly expressed in zinc-containing neurons in the brain and is particularly abundant in the hippocampus. Thus, MT-III is likely to play an important role in neuromodulation by zinc-containing neurons (57). Injection of zinc or copper intracerebroventricularly in rats increased MT levels in some areas of the brain (58), and 6-hydroxydopamine, a free radical generator, increased MT contents in brain cells (59).

These studies demonstrate that MT is induced by and can result in protection against environmental toxic insults, particularly oxidative injuries, in multiple organ systems. Several studies have shown that these agents can also induce MT expression in the heart (60–62), although the significance of this induction in protection against oxidative stress has not been addressed. In fact, toxicological studies on the heart have not been undertaken extensively. Nevertheless, it has long been recognized that certain chemicals induce cardiac injury, and reactive free radicals are critically involved in many cardiovascular diseases.

Myocardial Antioxidant and Oxidative Injury

Myocardial oxidative injury has long been known to play a major role in several cardiac pathophysiologic derangements, including cardiotoxicity induced by environmental chemicals and therapeutic drugs, ischemia-reperfusion injury, and heart failure resulting from multifactorial manifestations. These heart disease conditions have been reproduced in animal models, in which the critical role of free radicals in the disease development has been evaluated extensively.

The contractile function of the heart dictates its high metabolic demand. This, in turn, requires the heart be equipped with a rich supply of mitochondria. The mitochondrial respiratory chain is the primary energy-releasing system in the cell. The energy generated during the transport of electrons through the respiratory chain is conserved in the phosphate-bond energy of ATP. A series of oxidation-reduction reactions are involved in the energy generation. Of special concern for oxidative heart injury is the potential formation of highly reactive oxygen species during electron transport. Accumulation of these toxic oxygen species can result in exacerbation of damage to the heart.

Cardiac mechanisms of cellular defense against free radicals including reactive oxygen species involve the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHpx), as well as GSH and vitamin E. In experimental animal models, including rats, mice, and rabbits, the activity of catalase in the heart is ≈ 2%–4% of the activity (units/gram wet weight or protein) found in the liver (63–65). The activities of both Cu,Zn-SOD and Mn-SOD in the heart are also lower than in the liver, the activities in the heart being about 20%–30% of the activities in the liver when normalized by the amount of protein (64, 66). The activity of GSHpx in the heart of rats is about 80% of that found in the liver (64, 66), whereas it is only about 10% relative to liver in mice (67). The concentration of GSH in the heart of rats and mice is about 10%–20% of that in the liver (64, 66, 68). These observations suggest that the weak antioxidant capacity in the heart is adequate under physiological conditions. Presumably, reactive oxygen species generated during electron transport can be scavenged sufficiently, and no oxidative injury results. However, it appears that the heart is not well equipped to deal with the burst of reactive oxygen species generated under oxidative stress conditions.

In an attempt to increase the endogenous antioxidant capacity and cardiac protection against oxidative injury, we have produced a cardiac-specific, catalase-overexpressing transgenic mouse model (69). Using this unique experimental tool, we tested the hypothesis that increased catalase activity provides cardiac protection against oxidative injury. Results showed that elevation of catalase in the heart indeed increases the defense capacity against oxidative injury in-

duced by doxorubicin (DOX) (69) and ischemia-reperfusion (70). It has been noted from these studies that to obtain maximum protection from DOX-induced oxidative injury, catalase activity in the heart had to be elevated to an optimum level (60- to 100-fold higher than normal). We have found that 200-fold elevation of catalase activity in the heart did not provide protection against DOX toxicity, and 500-fold elevation might even enhance the toxicity of DOX. A similar observation has also been reported *in vitro*: transfection of L cells with a human catalase cDNA elevated catalase activity 100-fold. However, these catalase-enriched cells were more sensitive, rather than resistant, to the cytotoxicity of paraquat, bleomycin, and DOX than the untransfected cells from which they were derived (71). It was speculated that overt expression of catalase may cause imbalance between SOD and catalase (72, 73), which may lead to accumulation of superoxide radicals due to regeneration of molecular oxygen from hydrogen peroxide and continued redox cycle of DOX. In addition, catalase is an iron-dependent enzyme so iron released from degradation of catalase may form a complex with DOX. It has been shown that an iron-DOX complex is toxic (74).

Catalase is an enzyme that metabolizes H_2O_2 in the cell but has no capacity to react with superoxide or hydroxyl radicals. The ability of this single enzyme to provide protection against oxidative injury is therefore questionable at best. On the other hand, MT may be a more effective antioxidant to function in the heart. To test this idea, we used our cardiac-specific, MT-overexpressing transgenic mouse model (75) to determine the effect of MT on cardiac oxidative injury induced by DOX, ischemia-reperfusion, and dietary copper restriction.

MT and Doxorubicin (DOX) Cardiotoxicity

DOX is one of the most important anticancer agents. It is a valuable component of various chemotherapeutic regimens of breast carcinoma and small cell carcinoma of the lung. In metastatic thyroid carcinoma, DOX is probably the best available agent. It is also an important ingredient for the successful treatment of Hodgkin's disease and non-Hodgkin's lymphomas. However, cardiotoxicity and drug resistance are significant problems in the clinical application of DOX. The severe cardiotoxic effect of DOX has been a major limiting factor for its effective use in the treatment of cancers (76). The proposed mechanism for the cytotoxic effects of DOX is the production of reactive oxygen species during its intracellular metabolism (77).

The pathways by which DOX stimulates the formation of reactive oxygen species have been studied extensively. One major pathway is the formation of a DOX-iron complex (74, 78). This complex reacts spontaneously to generate hydrogen peroxide and hydroxyl radicals that cause oxidative damage (74, 78). Dexrazoxane (ICRF-187, ADR 529) reacts directly with the DOX-iron complex to promote the opening of its amide ring with a simultaneous transfer of the iron from DOX to the carboxylamine generated by the ring opening (79). This compound has been studied both experimentally and clinically for its potential as a cardioprotective agent (80). Limited protection against DOX cardiotoxicity with this agent has been observed, but it has never been sufficient (81). Most likely, this is partly due to other important pathways of reactive oxygen species generation by DOX.

The flavin reductases, including cytochrome *P*-450 reductase, cytochrome b_5 reductase, NADH dehydrogenase, and xanthine oxidase, all have the capacity to reduce DOX to DOX semiquinone free radical (82). In the presence of oxygen, the DOX semiquinone reacts rapidly to reduce the oxygen to superoxide, with regeneration of intact DOX. The superoxide is then converted to hydrogen peroxide, which is in turn converted to hydroxyl radical. The DOX semiquinone also reacts with hydrogen peroxide to yield hydroxyl radicals. A recent study has demonstrated another pathway, in which DOX binds to the endothelial isoform of nitric oxide synthase (eNOS) and undergoes eNOS-mediated reduction to become the semiquinone radical (83). As a consequence, superoxide formation is enhanced, and nitric oxide production is decreased. This may lead to generation of peroxynitrite and hydrogen peroxide, both of which are further converted to hydroxyl radicals. Neither of these two pathways of generation of reactive oxygen species by DOX is sensitive to the action of iron chelators.

A few animal studies have been undertaken to explore whether MT can provide protection against DOX cardiotoxicity. Preinduction of MT by bismuth subnitrate in mice has been shown to decrease DOX-induced lipid peroxidation in the heart (84). Zinc, cadmium, cobalt and mercury all induced MT expression in the heart and decreased DOX-related myocardial lipid peroxidation (84). The decrease in drug toxicity was dependent on the level of cardiac MT (85). In addition, pretreatment with bismuth subnitrate was necessary to protect mice against lethal doses of DOX, its co-administration with the drug having no effect (86). Under these circumstances, the DOX-induced production of conjugated dienes and malondialdehydes in the heart was negatively correlated with the concentration of MT in the tissue (86).

The studies, designed to assess the potential for MT to protect against DOX cardiotoxicity, were based on the understanding that MT is able to scavenge reactive oxygen species or to inhibit their formation. These studies employed agents to induce MT. However, as discussed earlier, these inducers were not specific for MT, and hence, cytoprotection may have been due to other pleiotropic effects. These could include activation or induction of other stress responses or repair systems, changes in cellular metabolic and transport processes, and delays in cell cycling. Factors such as stress proteins, GSH, and GSH-related antioxidant systems are all inducible by these agents and have been shown frequently to be involved in resistance to oxidative injury. In addition, these agents are not tissue-specific. Detoxification may occur in other tissues, such as the liver, in which MT is highly inducible. The observed de-

creased cardiotoxicity may be related to metabolic changes and/or pharmacokinetic shift of the drug. Taken together, the nature of these experiments precludes straightforward interpretation.

To overcome these problems, we developed the cardiac-specific, MT-overexpressing transgenic mouse model (75). MT was constitutively overexpressed in the heart only, and other antioxidant components including GSH, GSHpx, GSH reductase, catalase, and SOD were not altered in the MT-overexpressing heart. Using our unique experimental model, we demonstrated that MT provides protection against DOX-induced cardiomyopathy as examined by light and electron microscopy (75). In addition, the MT-overexpressing transgenic mice displayed markedly reduced bursts of serum creatine phosphokinase activity that was presumed to originate from the heart due to DOX-induced damage (75). Furthermore, DOX-induced functional deterioration was significantly inhibited in the isolated MT-overexpressing transgenic atrium relative to nontransgenic (75).

To further determine unequivocally the role of MT in cardiac protection against DOX, we have established for the first time a primary neonatal mouse cardiomyocyte culturing system (87). Ventricular cardiomyocytes, isolated from 1- to 3-day-old neonatal transgenic mice with high levels of cardiac MT and from nontransgenic controls, were used. On the 6th day of culturing, MT concentrations in the transgenic cardiomyocytes were about 2-fold higher than in the nontransgenic cells. DOX was added directly into the cultures. As compared to nontransgenic controls, transgenic cardiomyocytes displayed a marked resistance to DOX toxicity, as measured by morphological alterations, cell viability, mitochondrial malmetabolism, and lactate dehydrogenase leakage (88). This cytoprotective effect of MT correlated with inhibition of DOX-induced lipid peroxidation (88).

As mentioned above, acquired drug resistance of tumor cells is another important impediment for the clinical use of DOX. Clinical trials are ongoing using buthionine sulfoximine (BSO) to deplete GSH content in tumors, whose elevation was found to contribute to the acquired drug resistance. However, BSO also decreases GSH content in the heart, enhancing DOX cardiotoxicity. Because MT provides cardiac protection against DOX, we hypothesized that MT can compensate for the loss of protection from GSH depletion in the heart. We used transgenic mice with cardiac MT concentrations about 20-fold higher than normal and nontransgenic controls to test this hypothesis. The animals were treated with BSO by i.p. injection at 5 mmol/kg, two times at a 12-hr interval, before treatment with DOX at a single dose of 15 mg/kg. Four days after the DOX treatment, cardiac GSH was depleted by 60% in both transgenic and nontransgenic mice. DOX-induced cardiotoxicity, as measured by blood levels of creatine kinase and malondialdehyde concentrations in the heart, was dramatically increased in the BSO-treated nontransgenic mice. This increase was completely inhibited in the BSO-treated transgenic mice (89). These results thus demonstrated that cardiac MT-overexpressing transgenic mice are resistant to BSO-enhanced DOX cardiotoxicity. Selective modulations of decreasing DOX resistance in tumors by BSO and of increasing cardioprotection by MT induction may provide an alternative approach to improved DOX chemotherapeutic efficacy.

The data obtained from these studies using the cardiac-specific, MT-overexpressing transgenic mice and the primary cultures of neonatal cardiomyocytes derived from these transgenic mice thus demonstrate that high levels of MT can protect the heart from oxidative injury induced by DOX. Interestingly, in cultured cardiomyocytes, a mere 2-fold elevation of this protein provides sufficient protection against DOX's toxicity, although this may not be true *in vivo*. On the other hand, it seemed that overt elevation of MT in the heart does not attenuate its protection against DOX toxicity that occurred with overt expression of catalase (69). It is also noteworthy that 10-fold elevation of MT in the heart confers equivalent protection against DOX toxicity *in vivo* as 130-fold elevation (75). In contrast, in the catalase-overexpressing transgenic mice, a "dose-dependent" protection within the range of effective activities (15- to 100-fold higher than normal) was observed.

MT and Myocardial Ischemia-Reperfusion Injury

Myocardial damage induced by ischemia-reperfusion of the heart has been proposed to be caused, at least in part, by the generation of reactive oxygen species (90, 91). However, direct evidence to support the role of free radicals in this myocardial injury has not been obtained. The most important indirect evidence supporting this hypothesis has been the cardioprotective effects of agents capable of inducing antioxidants such as GSHpx (92) and SOD (93), and the beneficial effects of supplementing antioxidants *in vivo* or *in vitro* (94). It is difficult to interpret these experimental findings because the agents do not necessarily affect the status of only one or two antioxidant systems. If circulating antioxidants are supplemented *in vivo*, it is difficult to maintain constant plasma antioxidant concentrations and to predict the target tissue concentrations accurately. Importantly, high molecular weight antioxidants such as GSHpx, SOD, and catalase are unlikely to be transported into intracellular compartments. To overcome the shortcomings of these earlier studies, we employed the unique cardiac-specific, MT-overexpressing transgenic mouse model to test the hypothesis that elevation of MT in the heart provides protection against ischemia-reperfusion injury.

We applied a Langendorff perfusion model to examine directly the effects of MT on ischemia-reperfusion-induced derangements of contractile activity of the heart, myocyte injury as estimated by creatine phosphokinase release, and cell death as measured by the size of infarct zone. The cardiac MT concentrations in the transgenic mice used for this study were 55.7 ± 6.2 μg/g tissue, about 10-fold higher than that in the nontransgenic control mice (5.9 ± 0.5 μg/g

tissue). The hearts isolated from the transgenic mice and nontransgenic controls were first equilibrated for 30 min, then subjected to 50 min of warm (37°C) zero-flow followed by a 60- or 90-min reflow. There was no significant difference in the developed contractile force between the transgenic and control hearts during the 30-min equilibration period. Myocardial contractile force was increased at the beginning of ischemia, then fell to zero by 10 min of ischemia. There was no significant difference in tension between transgenic and control hearts during ischemia. The transgenic hearts, however, showed significantly better postischemic recovery of the suppressed contractile force (95).

Creatine phosphokinase activity in the collected perfusion effluent samples was measured. This activity from the samples during the preischemic equilibration period was undetectable in either group. Upon reperfusion, a high creatine phosphokinase activity was detected in the effluent collected from nontransgenic mouse hearts. A much lower activity was detected in the effluent collected from the transgenic mouse hearts; the peak value was about one-third of that from controls (95). The zone of infarction induced by a 90-min reperfusion following 50 min of ischemia was suppressed by about 40% in the transgenic hearts (95).

In the United States acute myocardial infarction, a consequence of myocardial ischemia, is the most common single cause of death. The treatment of this condition has been significantly improved by procedures allowing rapid return of blood flow to jeopardized myocardium (96). However, if a prolonged coronary occlusion results in severe myocardial infarction, the efficacy of these procedures significantly diminishes (96, 97). Therefore, any intervention that could avoid the onset of infarction would benefit patients.

To test if MT reduces the extent of myocardial infarction and to extend the results obtained from the Langendorff perfused hearts, we successfully adapted an *in vivo* open chest model of cardiac ischemia-reperfusion in mice (98, 99). In this model, cardiac ischemia was achieved by occluding the left coronary artery and reperfusion followed by releasing the occlusion. Using this newly established experimental procedure, we observed that the MT-overexpressing transgenic hearts were highly resistant to myocardial infarction induced by ischemia-reperfusion.

Based on these observations, we speculate that MT may be useful in the prevention of acute myocardial infarction. In particular, MT is highly inducible under a wide diversity of stress conditions, including oxidative stress. The regulation of MT expression has been well studied, and several agents, such as bismuth subnitrate (55), tumor necrosis factor-α (61), and isoproterenol (62) have been identified to elevate MT levels selectively in the heart. Therefore, the basis for developing pharmaceutical agents to increase MT concentration in the heart already exists. Exploring the potential for MT to protect against cardiac ischemia-reperfusion injury would likely result in novel approaches to myocardial ischemic disease and could positively influence clinical outcomes.

MT and Heart Failure

Heart failure represents a convergent myocardial pathological derangement resulting from a number of primary cardiac disorders. Although the pathological mechanisms leading to heart failure are unknown, data from animal studies have suggested that increases in free radical formation and subsequent oxidative stress are involved in the development of heart failure (100, 101). Human studies also have demonstrated that plasma malondialdehyde concentrations are increased, along with decreased plasma thiol concentrations, in patients with chronic congestive heart failure (102). To determine the role of free radicals in heart failure, the MT-overexpressing transgenic mouse model provides a unique experimental approach.

Several studies using rat and guinea pig models have successfully produced many pathophysiologic aspects of heart failure (100, 103, 104). In these studies, the left coronary artery was ligated 1–2 mm from its origin. This procedure produced about 35% mortality within 24 hr after ligation. The surviving animals developed heart hypertrophy postacute myocardial infarction. However, the same procedure cannot be applied to the mouse. Coronary artery ligation results in more than 50% mortality, and the surviving mice live no more than 3 weeks, insufficient time for heart failure to develop. Therefore, an alternative mouse model of heart failure needed to be developed.

Dietary copper (Cu) restriction causes cardiac hypertrophy followed by heart failure in rodent models. A great deal of similarity has been observed in cardiomyopathy induced by Cu deficiency and that induced by pressure overload (105). We have recently demonstrated that the molecular aspects of myocardial remodeling resulting from the two different etiologies are also similar (Kang YJ, Wu HY, Saari JT, unpublished data). The unique aspects of Cu-deficient mice are that 1) the mortality prior to heart failure is very low (less than 5%); 2) the survival time is sufficient to allow detailed studies of the development of heart failure; and 3) the unified feeding treatment significantly reduces the artifact due to coronary artery ligation surgery.

Therefore, we have used the cardiac-specific, MT-overexpressing transgenic mouse model to study the effect of MT on Cu deficiency–induced heart failure. The transgenic mice were bred with the same strain (FVB) nontransgenic mice. Dams of the pups (both transgenic mice and their nontransgenic littermates) were fed a Cu-deficient or a Cu-adequate control diet starting on the fourth day postdelivery, and the weanling mice were continued on the same diet until they were sacrificed. After these animals were placed on the Cu-deficient diet for 3 weeks, systemic Cu deficiency developed equally in both transgenic mice and nontransgenic controls, as determined by markedly decreased cardiac and hepatic Cu concentrations, lowered

plasma ceruloplasmin concentrations, and suppressed Cu,Zn-SOD activities in the liver.

The extent of cardiac hypertrophy and its progression were determined in transgenic and control mice. MT concentrations in transgenic mouse hearts were about 40-fold higher than in nontransgenic. Both were hypertrophic after these animals were placed on the Cu-deficient diet for 4 weeks. This hypertrophy further developed in the nontransgenic mouse hearts as the feeding continued. In contrast, the progression of heart hypertrophy was inhibited in the transgenic mice, and this inhibitory effect of MT was correlated with its suppression of lipid peroxidation (106).

Progression of heart hypertrophy leads to heart failure, which is associated with elevations in some molecular markers. We have examined the expression of atrial natriuretic peptide (ANP) in the left ventricle of Cu-deficient mice. The ANP gene is expressed in both atrium and ventricle during embryonic development, but its expression is downregulated in the ventricle shortly after birth, leaving the atrium as the primary site of ANP synthesis within the mature myocardium. During the development of heart failure, reexpression of ANP in myocytes of the left ventricle occurs (107). Measurement of the relative abundance of ANP mRNA in the left ventricle of mice placed on a Cu-deficient diet for 5 weeks revealed that Cu deficiency caused a significant elevation of ANP mRNA in the left ventricle, which was markedly depressed in the MT-overexpressing transgenic mice.

Recent progress in myocardial research has provided significant insight into cellular mechanisms of heart failure. It has been recognized that the loss of cardiac myocytes is a fundamental part of the process that initiates and/or aggravates heart failure and leads to premature death (108–111). An important aspect of the loss of myocytes is that it occurs *via* apoptosis (112–116), as has been demonstrated in the myocardium in heart failure patients (115, 116). We have examined apoptosis in the hearts of Cu-deficient mice using a TUNEL assay. It has been estimated that in the Cu-deficient nontransgenic hearts, there was about 0.5% cardiac cell populations undergoing apoptosis per day. This apoptotic effect of Cu deficiency was significantly inhibited in the MT-overexpressing transgenic hearts (only less than 0.1% apoptotic cardiac cells per day). It is important to stress that the apoptosis observed in the Cu-deficient heart would lead to very significant consequences in terms of the loss of myocytes. In a carefully designed time-course study (117), it has been estimated that a cardiomyocyte may traverse apoptosis in less than 20 hr in rats. If apoptosis occurs at the constant rate (about 0.5% myocytes per day by the Cu-deficient diet feeding as shown above), the overall loss of myocytes due to apoptosis would be remarkable after a certain period. For example, over 5 weeks, a total of 17%–18% of total cardiomyocytes may be lost due to Cu deficiency. This estimate is based on the fact that adult myocytes do not regenerate, and once a myocyte undergoes apoptosis, it will be eliminated from the myocardium.

It has been demonstrated that ANP induces apoptosis in cardiomyocytes (118). A correlation between induction of ANP expression and apoptosis in the heart from Cu deficiency has been observed in our studies described above. MT inhibits not only ANP expression but also apoptosis induced by Cu deficiency. Taken together with the inhibitory effect of MT on progression of Cu deficiency–induced heart hypertrophy, the data strongly indicate that MT inhibits the development of heart failure in the Cu-deficient mice. Because MT also inhibits Cu deficiency–induced lipid peroxidation in the heart, it is possible that the accumulation of free radicals is critically involved in the development of heart failure and that MT, by preventing this accumulation, inhibits the free radical–mediated development of heart failure.

Possible Mechanisms of MT Protection Against Oxidative Injury

Studies *in vitro* have demonstrated that MT reacts directly with all reactive oxygen species (40–42). However, it is questionable whether these *in vitro* observations are applicable to the *in vivo* action of MT. In particular, all of the reactive oxygen species, especially hydroxyl radicals, are very reactive and have an extremely short half-life. It is speculated that MT can only be effective as a free radical scavenger *in vivo* if it is located sufficiently close to the site of production of the radicals to interact with them before their reaction with other cellular components. Depending on the local concentrations of MT, this may predict that the direct interaction between MT and the radicals as a major mechanism of action *in vivo* is impracticable.

It has been demonstrated that the cluster structure of Zn-MT provides a chemical basis by which the cysteine ligands can induce oxidoreductive properties (33). This structure allows for thermodynamic stability of zinc in MT while permitting zinc to retain kinetic lability. This is demonstrated by the fast zinc exchange between MT isoforms (28), between MT and the zinc cluster in the Gal4 transcription factor (28), and between MT and the apoforms of various zinc proteins (27, 31). Importantly, zinc release from MT is modulated by both GSH and GSH disulfide (GSSG) (24, 31, 32). GSH inhibits zinc release in the absence of GSSG, indicating that MT is stabilized at relatively high cellular GSH concentrations. The presence of GSSG, or any other oxidizing agent, results in a release of zinc that is synergistically increased by GSH. The rate of zinc release depends linearly on the amount of GSSG (i.e., the more oxidative the redox state becomes, the more efficiently zinc is released from MT) (27). It has long been known that zinc transfer from MT to other proteins actually occurs *in vivo* (119). Moreover, cellular disulfides other than GSSG also react with MT to release zinc with high efficiency (24).

Mobilization of zinc from MT by an oxidative reaction may either constitute a general pathway by which zinc is distributed in the cell, or it may be restricted to conditions of stress where zinc is needed in antioxidant defense sys-

tems (24). It has been argued that the primary determinant of MT protection against oxidative stress is the release of zinc sequestered by MT and its subsequent uptake by plasma membranes, since zinc protects against lipid peroxidation and thereby stabilizes membranes (42, 120). In addition, released zinc may suppress lipid peroxidation by affecting many different cellular functions, such as decreasing iron uptake and inhibiting NADPH-cytochrome-*c* reductase (121). We have observed that zinc concentrations in the MT-overexpressing transgenic mouse hearts are significantly increased.

If the reaction between Zn-MT and disulfides triggers a mechanism of Zn release and the cardiac protection by MT against oxidative injury is mediated by the released Zn, a dynamic change in the level of Zn bound to MT during oxidative stress would occur. In conjunction with this change, MT would become oxidized. These events have not been observed in intact animals, and experiments to test this idea are not straightforward. However, it is crucial to elucidate these mechanisms to understand the comprehensive antioxidant action of MT *in vivo*. With the advances in technology and recently developed state-of-the-art approaches, novel insights into the biological function of this unusual and ubiquitous protein are on the horizon.

Controversial Issues

A study using transgenic mice overexpressing MT in multiple organs has shown that cardiac MT levels were increased by about 3-fold. No protection from DOX cardiotoxicity was observed in these mice (122). In this study, cardiotoxicity was assessed by survival, fluid accumulation, and lipid peroxidation. It was concluded that elevated cardiac MT does not necessarily protect against DOX cardiotoxicity. Perhaps, MT concentrations in these transgenic mice were not high enough to be effective. Therefore, it is not only a matter of whether MT is involved in this cardioprotection, but also the extent of MT elevation in the heart required for protection. In our studies using cardiomyocytes isolated from the cardiac-specific MT-overexpressing transgenic mice, we observed that even a 2-fold elevation of MT provided marked resistance to the toxicity of DOX.

It is critically important that we quantitate the distribution of the elevated MT among different cell types in the heart *in vivo*. MT was found in the cardiomyocyte isolated from the cardiac-specific, MT-overexpressing transgenic mice produced in this laboratory (88). Therefore, an important distinction of our work is that we have shown the overexpressed MT to be targeted to the cardiomyocyte, a key cell type.

Not only does MT cellular distribution affect the action of MT, but also it is known that MT subcellular localization may significantly interfere with the effectiveness of MT protection against oxidative injury. Overexpression of cytoplasmic MT did not protect against *tert*-butyl hydroperoxide-induced DNA damage, but it provided protection against its cytotoxic effects in NIH 3T3 cells (48). Therefore, it is critical to understand the end points to be examined and the potential effects of MT on these parameters in the context of not only its effective concentrations, but also its action sites.

The aforementioned conflict regarding the effect of MT on DOX cardiotoxicity raises other issues. Mortality as an end point (122) does not necessarily reflect cardiotoxicity. In fact, it has not been documented that cardiotoxicity of DOX correlates with mortality, particularly at the lethal dose of the drug. Likewise, peritoneal fluid accumulation is not a specific end point of, and may be independent of, cardiotoxicity (122).

Specific measurements of cardiac oxidative injury by DOX were performed by examining the concentrations of 4-hydroxy-2-(E)-nonenal and malondialdehyde in the previous study (122). MT transgenic mouse hearts actually displayed higher lipid peroxide concentrations relative to nontransgenic mice. No explanation for this unexpected observation was offered. An important caveat in using these byproducts as indicators of lipid peroxidation is that their rate of formation is highly inefficient and varies according to the transition metal ion content of the sample (123). Yet MT binds metals, and the composition of transition metal ions in MT may interfere with the measurement. On the other hand, the metal composition in MT critically affects the action of this protein. In addition to Zn partitioning, Cd-Zn-MT actually induces DNA strand breaks *in vitro* (124), and Cu-MT is protective against oxidative stress in yeast (125). Therefore, the metal speciation of MT is critically determinative in the action of MT.

Another observation that has often been reported is the lack of a correlation between MT induction and cytoprotection against oxidative injury. We have observed that treatment of mice with a single 15 mg/kg ip dose of DOX for 4 days markedly induced MT synthesis in the heart. The MT protein concentration in the heart was increased about 4-fold, and the MT mRNA abundance was elevated about 25-fold (126). However, DOX caused severe cardiac toxic effects, as assessed by cardiomyopathy examined by electron microscopy, elevation of serum creatine phosphokinase activity that presumably resulted from the injured heart, and myocardial lipid peroxidation (75). On the other hand, under the same experimental conditions, the transgenic mice with constitutively elevated concentrations of MT were significantly resistant to the cardiotoxicity of DOX (75). These results suggest that 1) MT may not provide the first line of defense against oxidative stress under physiological conditions; 2) induction of endogenous MT under oxidative stress conditions may occur as a consequence of oxidative injury, when it may be too late for MT to protect against the injury; and 3) MT may prevent further oxidative injury but may not repair the injury that occurred prior to its induction. Therefore, it is important to examine experimental details critically in the context of pre-existing conditions to interpret the results, and conclusions should not be derived only from a simple correlation analysis.

Conclusions

Despite more than 40 years of efforts in search of the biological function of MT, answers remain elusive. In spite of significant technological advances ranging from pharmacologic inducers to transgenic mice, the only consensus among MT researchers is that MT has a role in detoxification of transition metals. The antioxidant function of MT was suggested in the early 1980s. Studies *in vitro* have indeed demonstrated a direct reaction between MT and reactive oxygen species (which react with almost everything). However, it has been debated whether MT functions as an antioxidant *in vivo* because the *in vitro* action of MT as a free radical scavenger has never been demonstrated in intact animal studies. In the last few years, studies using cardiac-specific, MT-overexpressing transgenic mouse models have produced direct evidence to support the antioxidant and protective function of MT from oxidative injury in the heart. The supporting data gathered from diverse experimental settings, including both acute and chronic oxidative stress conditions, are compelling. However, the debate concerning the antioxidant function of MT *in vivo* cannot be settled until a comprehensive understanding of the mechanism of action of MT is obtained. With the advances in molecular biotechnology and an understanding of the zinc cluster structure of MT, it is foreseeable that novel insights into this problem are forthcoming.

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