Inhibition of Doxorubicin Toxicity in Cultured Neonatal Mouse Cardiomyocytes with Elevated Metallothionein Levels

GUANG-WU WANG and Y. JAMES KANG
Department of Medicine, University of Louisville School of Medicine, Louisville, Kentucky
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ABSTRACT

Controversial results have been reported regarding whether metallothionein (MT) functions in doxorubicin (DOX) detoxification in the heart. To determine unequivocally the role of MT in cardioprotection against the toxicity of DOX, ventricular cardiomyocytes isolated from 1- to 3-day neonatal transgenic mice with high levels of cardiac MT and from nontransgenic control animals were applied. On the 6th day of culturing, MT concentrations in the transgenic cardiomyocytes were about 2-fold higher than those in the nontransgenic cells. DOX was added directly into the cultures. Compared with nontransgenic controls, transgenic cardiomyocytes displayed a significant ($p < .05$) resistance to DOX cytotoxicity, as measured by morphological alterations, cell viability, and lactate dehydrogenase leakage from the cells. This cytoprotective effect of MT correlated with its inhibition of DOX-induced lipid peroxidation. These observations demonstrate unequivocally that elevation of MT concentrations in the cardiomyocytes of 2-fold higher than normal provides efficient protection against DOX toxicity.

Cardiotoxicity is a major factor that limits the clinical usefulness of doxorubicin (DOX) (Buzdar et al., 1985). DOX is one of the most powerful anticancer agents that is effective in the treatment of acute leukemias and malignant lymphomas, as well as a number of solid tumors (Blum and Carter, 1974). The cardiotoxicity is believed to result mostly from the reactive oxygen species produced during the intracellular metabolism of this drug (Myers et al., 1977). Therefore, a number of efforts have been made to apply free radical scavengers to protect the heart from DOX-induced damage (Van Vleet et al., 1980; Dimitrov et al., 1987; Balanerhu and Nagarajan, 1992). Several experimental approaches using exogenously supplemented antioxidants (Myers et al., 1983; Unverferth et al., 1985), however, have achieved limited successes due to some shortcomings: 1) it is impossible to maintain constant plasma antioxidant concentrations and to accurately predict the target tissue (heart) concentrations, 2) activation and inactivation by multiple metabolic organ systems such as liver and kidney would greatly affect the efficacy of the antioxidants in the heart, and 3) high-molecular-weight antioxidants such as catalase and superoxide dismutase are unlikely to be transported into intracellular compartments.

These limitations require new experimental approaches to improve the application of free radical scavengers in reducing DOX cardiotoxicity.

Recent studies have shown that metallothionein (MT) plays an important role in scavenging free radicals, thereby preventing oxidative injury (Sato and Bremner, 1993). The most intrinsic aspect of MT in the potential for this application in vivo is its inducibility by a variety of physical and chemical stressors. It has been shown that MT can be induced to a significantly high level in multiple organ systems by metals, adrenocortical steroids, cytotoxic xenobiotics, cytokines, and many other stress-producing conditions (Naganuma et al., 1985; Satoh et al., 1988; Iszard et al., 1995). The gene regulation of MT is complex (Andrews, 1990). A wide range of transcription factors, including the metal regulatory element, glucocorticoid-responsive element, and interferon-related element, can interact with the MT promoter regions. Because of the high inducibility of MT, many studies have been undertaken to use different inducers to examine its role in a variety of cellular processes. For instance, bismuth subnitrate has been used to increase MT concentrations in the heart and other organs of mice (Naganuma et al., 1988). The bismuth subnitrate-pretreated mice were significantly resistant to cardiotoxicity induced by subsequent treatment with DOX. This resistance was highly correlated with the cardiac MT concentrations.

We have produced a transgenic mouse model in which MT
was overexpressed specifically in the heart (Kang et al., 1997). Using this unique experimental model, we demonstrated that DOX-induced morphological changes in the myocardium and creatine kinase release from the heart were significantly inhibited (Kang et al., 1997). However, a study using transgenic mice in which MT was overexpressed in multiple organs, including the heart, has shown that MT did not provide protection against DOX cardiotoxicity (DiSilvestro et al., 1996). This controversial result indicates that further studies are necessary to directly examine the effect of MT on cardiac oxidative injury, particularly when the potential for the clinical application of MT inducers in protection against DOX cardiotoxicity should be considered.

To this end, we recently established a primary neonatal cardiomyocyte culture system. This cell culture model was applied in the present study to define directly the role of MT elevation in cardiac protection against DOX toxicity. Morphological alterations, lactate dehydrogenase (LDH) leakage, cell viability, and lipid peroxidation were compared between DOX-treated transgenic cardiomyocytes and nontransgenic controls. All of the results clearly demonstrate that MT elevation provides cardioprotection against DOX toxicity.

**Experimental Procedures**

**Animals.** FVB mice obtained from the University of Louisville Research Resources Center were housed in the animal quarters and maintained at 23°C with a 12-h light/dark cycle. They were given free access to rodent chow and deionized water. Transgenic mice overexpressing MT specifically in the heart were produced from the FVB strain. Detailed descriptions of the development and characterization of these transgenic mouse lines were reported previously (Kang et al., 1997). These animals were maintained under the same conditions as described above. The transgenic founder mice were bred with nontransgenic mice of the same strain. The resultant litters were identified by a pigment marker (dark eye and fur) at birth. This pigment transgene was co-injected with the MT transgene into the early embryo when the transgenic mice were produced. Both transgenic positive (heterozygotes) and negative neonatal mice were used for experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

**Materials.** Eagie's minimum essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) without phenol red, fetal bovine serum (FBS), and trypsin were purchased from Gibco BRL (Grand Island, NY). The bicinchoninic acid protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL). DOX and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were obtained from Sigma Chemical Co. (St. Louis, MO). The lipid hydroperoxide assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals were purchased from Fisher Chemical Co., Sigma Chemical Co., or Aldrich Chemical Co. (Milwaukee, WI). All reagents were of at least analytical grade.

**Neonatal Mouse Primary Cardiomyocyte Culturing.** A new procedure for culturing ventricular cardiomyocytes from neonatal mouse was established through modifications of the methods used for neonatal rat (Goldspink et al., 1996; Long et al., 1997) and fetal mouse (Goshima, 1976; Nakamura et al., 1993) cardiomyocyte cultures. The 1- to 3-day-old neonatal transgenic or nontransgenic mice were sacrificed by cervical dislocation. Hearts were removed aseptically (retaining the ventricles only) and maintained in cold Hank's balanced salt solution (HBSS) without Ca**++** and Mg**++**, pH 7.4. The cells from subsequent digestion were added to an equal volume of cold HBSS with Ca**++** and Mg**++**, pH 7.4, until all cardiac cells were isolated. The resulting mixture was centrifuged for 8 min at 200g, and the cells were resuspended in the FBS-MEM [MEM supplemented with 20% FBS (v/v), 100 units/ml penicillin, and 100 μg/ml streptomycin]. To exclude non-muscle cells, the isolated cells were first plated onto tissue culture dishes at 37°C for 2 h under a water-saturated atmosphere of 5% CO₂ with 95% air, based on the observation that nonmuscle cells attach to the substrata more rapidly (Polinger, 1970). The suspended cells were then collected and plated at a density of 1.0 × 10⁵ cells/cm² and incubated under the same conditions as above. Myocyte purity was monitored by staining with antibody to cardiac-sarcomeric actin according to the manufacturer’s instructions (Sigma Chemical Co.). Myocyte purity averaged 94 ± 5% when examined at 48 h after culturing.

**Cellular MT Concentration.** Total MT was determined with the cadmium-hemoglobin affinity assay (Eaton and Cherian, 1991). Cells were harvested after being precultured for 2 h or on the 6th day of postculturing. The cells were rinsed with 5 ml of cold PBS and centrifuged at 2000g for 10 min; 500 μl of 10 mM Tris-HCl was added to the pellet. The cells then were pulse-sonicated on ice with a dismembrator (model 60; Fisher Scientific Inc.) at an output power of 8 for 15 s repeated three times with a 30-s interval. After centrifugation at 10,000g for 15 min, 200 μl of supernatant was transferred to microtubes for total protein determination using the Pierce Chemical Co. bicinchoninic acid protein assay reagents (Smith et al., 1985), with bovine serum albumin as the standard.

**Determination of DOX Cytotoxicity.** The cytotoxicity of DOX was determined by examining morphological alterations of the cardiomyocytes, measuring LDH release from the cells, and monitoring cell viability. To observe cell morphological alterations, 6-day-old cultures were treated with 0.001, 0.01, 0.05, 1.0, 2.0, or 4.0 μM DOX. At the time of DOX exposure, the FBS-MEM was removed and replaced with fresh serum-free MEM containing the desired concentrations of DOX. Toxicities were evaluated 24, 48, and 72 h after the primary cell cultures were treated with DOX. A Zeiss inverted phase-contrast microscope was used to observe cell morphology as described previously (Melchert et al., 1991). Photomicrographs were taken at 10× magnification using a 35-mm Canon camera attached to the microscope. Morphological alterations were classified as 1) pseudopodia, or extension or retraction of the cell membrane; 2) vacuoles, or the appearance of clear inclusion bodies of cytoplasmic materials; or 3) granules, or the appearance of dark granular materials. Gross cellular morphological alterations were evaluated with a grading scale: NC indicates no obvious changes; +, minimal alterations; ++, intermediate alterations, and ++++, extensive alterations.

The activity of cytoplasmic enzyme LDH that was released into the culture media was determined with the method described previously (Wroblewski and LaDue, 1955). On the 6th day of culturing, cardiomyocytes were treated with the same concentrations of DOX, and the same protocol was followed as described for the morphological examination. A 100-μl sample from the culture media was collected after the cells were treated for 6, 9, 24, 48, or 72 h, and the LDH activity was measured in 2.4 ml of phosphate buffer (0.1 M, pH 7.4) with 100 μl of Na-pyruvate (2.5 mg/ml phosphate buffer) and 100 μl of NADH (2.5 mg/ml phosphate buffer). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm at 25°C using a spectrophotometer (model DU-650; Beckman Instruments; Columbia, MD).

Cell viability was determined by a short-term microculture tetrazolium (MTT) assay (Welder et al., 1991). In 96-well microplates, 2.5 × 10⁴ cells/well were incubated in 100 μl of culture media for 48 h. The cells were exposed to different concentrations of DOX for 3 h. The media containing DOX were replaced by fresh media without DOX, and the cells were incubated for an additional 48 h. The
media were removed again and replaced with 90 \mu l of DMEM (containing no phenol red or FBS) and 10 \mu l of MTT solution (2 mg/ml phosphate buffer) for 4 h. After the MTT-containing DMEM was removed, the remaining formazan blue crystals were dissolved in 75 \mu l/well of a 0.04 N HCl/isopropanol alcohol mixture. Absorbance at 540 nm was measured using a microplate reader (model EL 311; Bio-Tek Instruments, Winooski, VT).

**Measurement of Lipid Hydroperoxide.** Lipid peroxidation is traditionally quantified by measuring malondialdehyde and 4-hydroxynonenal. These assays are nonspecific and often lead to miscalculation of lipid peroxidation. A new lipid hydroperoxide assay kit (Cayman Chemical Co.) was used that measured the hydroperoxide concentration by directly using the redox reactions with ferrous ions. The extraction procedure and measurement of the extracted lipid hydroperoxides were performed according to the manufacturer’s instructions.

**Statistical Analysis.** Data were analyzed initially by one-way analysis of variance. Scheffe’s F test was used for further determination of the significance of differences. Differences between MT-overexpressing transgenic cardiomyocytes and nontransgenic controls were considered significant at p < .05. The data are presented as mean ± S.D. values from triplicate cultures for each treatment.

**Results**

Cultured cardiomyocytes isolated from transgenic neonatal mice and nontransgenic control animals showed the same characteristics as examined by morphological, biochemical, and functional features (Wang et al., 1999). Antioxidant activities in these cells changed during culturing, as shown in Table 1. However, there was no significant difference in any of these changes between transgenic and nontransgenic cultures. Total MT concentrations in the hearts of transgenic and nontransgenic neonatal mice were 20.93 ± 5.80 and 0.49 ± 0.06 \mu g/mg protein, respectively. After the cardiomyocytes were isolated and precultured for 2 h, MT concentrations were dramatically decreased in the transgenic cells, being 3.45 ± 1.38 \mu g/mg protein (0.45 ± 0.19 \mu g/mg protein in the nontransgenic cells). Further decrease was observed after the transgenic cultures were cultured for 6 days, being 1.01 ± 0.13 \mu g/mg protein (0.44 ± 0.09 \mu g/mg protein in nontransgenic cells). The MT concentrations in the transgenic myocardiocytes, however, remained constantly significantly higher than those in the nontransgenic cells.

The effect of MT elevation on DOX-induced morphological alterations was examined, as illustrated in Fig. 1 and summarized in Table 2. DOX induced a dose- and time-dependent detrimental effect on the structures of both transgenic and nontransgenic cardiomyocytes. However, the transgenic cells were much more resistant to this toxic effect. At the DOX concentration of 0.01 \mu M in the cultures, nontransgenic cell cultures displayed monolayer disruption after treatment for 48 h, whereas the transgenic cultures did not show such a change. The same contrast was observed after these two types of cells were exposed to 0.1 \mu M DOX for 24 h. At higher DOX concentrations, the transgenic cardiomyocytes showed destructive appearance. However, the severity was much less than that observed in the nontransgenic cells.

When the cytotoxicity was examined by the release of LDH from the cells into media, a typical dose- and time-dependent effect of DOX was observed in both types of cells. There was no significant difference in the cellular LDH leakage between the two types of cells untreated with DOX. With treatment of 2.0 \mu M DOX for more than 48 h, the LDH release from the nontransgenic cells was significantly increased. This did not occur in the transgenic cells (Fig. 2). The same result was obtained when these cells were exposed to varying concentrations of DOX for 72 h (Fig. 3). The LDH released from the nontransgenic cells again was much more than that from the transgenic cells at higher concentrations applied. To confirm the above results, the LDH activities in the cells were determined. As shown in Fig. 4, significant (p < .05) higher activities of LDH remained in the transgenic cells, in agreement with the results obtained from the analyses of LDH leakage. However, the determination of intracellular LDH was more sensitive and indicative for the DOX cytotoxicity.

The effect of DOX on cell viability was determined with the MTT assay as described in Experimental Procedures. At low concentrations (less than 0.1 \mu M), DOX did not cause a significant detrimental effect on either type of cell. At concentrations of more than 0.1 \mu M, DOX showed a toxic effect on both types of cardiomyocytes, whereas the transgenic cells were much more resistant to this effect (Fig. 5).

Finally, we tested whether the observed protection by MT elevation against DOX cytotoxicity could correlate with its inhibitory effect on lipid peroxidation induced by this drug. The lipid hydroperoxide concentrations in the cells were estimated as shown in Fig. 6. There was no significant difference in cellular lipid hydroperoxide concentrations between these two types of cardiomyocytes untreated with DOX. After these cells were treated with 2.0 \mu M DOX for 24 h, lipid hydroperoxide concentrations were significantly increased in the nontransgenic cells but were not changed in the transgenic cells.

**Table 1**

Changes of glutathione concentrations and antioxidant activities in cultured nontransgenic (control) and transgenic cardiomyocytes with culturing time.

<table>
<thead>
<tr>
<th></th>
<th>Preculture</th>
<th>6-Day Culture</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MT-TG</td>
</tr>
<tr>
<td>Glutathione (nmol/mg protein)</td>
<td>0.40 ± 0.05</td>
<td>0.45 ± 0.22</td>
</tr>
<tr>
<td>Glutathione peroxidase (nmol NADPH/min/mg protein)</td>
<td>24.08 ± 5.82</td>
<td>28.35 ± 4.29</td>
</tr>
<tr>
<td>Catalase (\mu mol H₂O₂/min/mg protein)</td>
<td>69.33 ± 40.27</td>
<td>85.56 ± 19.66</td>
</tr>
<tr>
<td>Superoxide dismutase, total (U/mg protein)</td>
<td>3.43 ± 1.35</td>
<td>3.76 ± 1.46</td>
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MT-TG, transgenic cardiomyocytes.

Values are mean ± S.D., n = 3–6.

* p < 0.05.
Discussion

The application of free radical scavengers to decrease DOX cardiotoxicity has been a favorite approach to improve the efficacy of this drug in cancer chemotherapy. In this context, MT is an excellent candidate for such an application. MT is a highly conserved, low-molecular-weight, thiol-rich protein. The mammalian MT has 61 amino acids, including 20 cysteine residues, but no aromatic amino acids or histidine or leucine. The basal level of MT in biological systems is very low, although it may vary with age and type of tissue. However, this protein is induced to a significantly high level when the system is challenged by heavy metals, starvation, heat, inflammation, or other stress conditions. Importantly, MT likely functions as an oxy-radical scavenger (Thornalley and Vasak, 1985). Zinc-MT has been shown to react with hydroxyl radicals in a cell-free system and to be more effective than glutathione in preventing hydroxyl radical-induced DNA degradation (Abel and de Ruiter, 1989). A recent study using HL-60 cells has demonstrated a direct reaction of hydrogen peroxide with the sulphydryl groups of MT (Quesada et al., 1996). Moreover, this study has shown that the thiolate groups in the MT were the preferential attacking targets of hydrogen peroxide compared with the other sulphydryl residues from glutathione and protein fractions.

The protective effect of MT on DOX cardiotoxicity was initially determined using a mouse model in which tumor cells were implanted (Naganuma et al., 1988). The oral administration of bismuth subnitrate significantly elevated
cardiac MT concentrations and decreased cardiotoxicity observed with a single subcutaneous injection of DOX. Interestingly, this bismuth subnitrate treatment did not affect the antitumor activity of DOX. This observation holds potential for the clinical application of MT inducers in improving DOX chemotherapeutic efficacy. However, it is important to confirm that the cardiac protection from DOX toxicity by these inducers indeed resulted from MT production. In our recent study (Kang et al., 1997), we produced transgenic mice in which cardiac MT was specifically overexpressed. These transgenic mice with MT concentrations in the heart about 10- or 130-fold higher than normal showed a significant resistance to DOX-induced cardiomyopathies, creatine phosphokinase release from the heart, and contractile functional depression of the heart. Furthermore, 10-fold elevation showed the same protective effect as that at 130-fold (Kang et al., 1997).

### TABLE 2

<table>
<thead>
<tr>
<th>DOX Concentration</th>
<th>Pseudopodia</th>
<th>Vacuoles</th>
<th>Granule</th>
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<tr>
<td></td>
<td>Control MT-TG</td>
<td>Control MT-TG</td>
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<tr>
<td>0.01 μM DOX</td>
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<tr>
<td>24 h</td>
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<td>48 h</td>
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<td>0.1 μM DOX</td>
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<td>0.5 μM DOX</td>
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<td>1.0 μM DOX</td>
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<td>2.0 μM DOX</td>
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<td>24 h</td>
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<tr>
<td>4.0 μM DOX</td>
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<td>48 h</td>
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Primary neonatal mouse cardiomyocyte cultures were maintained in culture for 6 days and then exposed to different concentrations of DOX for varying times. MT-TG, transgenic cardiomyocytes; NC, no change; +, minimal alterations; ++, intermediate alterations; ++++, extensive alterations; —, cells disappear.

**Fig. 2.** Time-dependent effect of DOX on LDH release from primary MT-overexpressing transgenic cardiomyocytes and nontransgenic controls. Cells were cultured for 6 days before exposure to 2.0 μM DOX. Values represent mean ± S.D. from triplicate samples for each treatment at each time point. *Significantly different from nontransgenic control at corresponding concentrations (p < .05).

**Fig. 3.** Dose-dependent effect of DOX on LDH release from primary MT-overexpressing transgenic cardiomyocytes and nontransgenic controls. Cells were cultured for 6 days before exposure to varying concentrations of DOX for 72 h. Values represent mean ± S.D. from triplicate samples for each treatment at different concentration levels. *Significantly different from nontransgenic control at corresponding concentrations (p < .05).

**Fig. 4.** Dose-dependent effect of DOX on leakage of LDH from MT-overexpressing transgenic cardiomyocytes and nontransgenic controls by measuring remaining LDH activities in the cells. Intracellular LDH activities were determined 72 h after cultures were exposed to varying concentrations of DOX. Triplicate samples were used for each determination. *Significantly different from nontransgenic control (p < .05).
It has been argued that a 10-fold or higher increase in cardiac MT concentrations may not be practical when MT inducers are used (DiSilvestro et al., 1996). A study using transgenic mice overexpressing MT in the heart less than 3-fold higher than normal did not show protection against DOX cardiotoxicity (DiSilvestro et al., 1996), which was assessed by survival, fluid accumulation, and lipid peroxidation. From this study (DiSilvestro et al., 1996), the authors concluded that high heart MT concentrations do not necessarily protect against DOX cardiotoxicity. It was first suggested that MT concentrations in the transgenic mice may not be high enough to be effective. However, the MT concentrations in the transgenic mice were at comparable levels to that in the bismuth subnitrate-treated mice. This leads to an alternative explanation that bismuth may act via the combination of MT induction plus other effects and that MT may not actually be involved in the bismuth action on DOX toxicity (DiSilvestro et al., 1996).

The issues related to MT protection against DOX cardiotoxicity are not only whether MT is involved in this cardioprotection but also how much MT elevation in the heart is required for this protection to occur. In the present study, the cardiomyocytes isolated from the MT-overexpressing transgenic mouse heart were used. Interestingly, MT concentrations in these transgenic cells were decreased to only about 2-fold higher than normal after they were cultured for 6 days, although the concentrations were about 40-fold higher in the neonatal heart before culturing. This unexpected observation suggests another aspect of MT metabolism. It seems that MT may be transported out of cardiomyocytes in proportion to the intracellular concentrations, or the cells may retain a threshold level of intracellular MT and that above this level, this protein would be surplus to other cells or extracellular matrix. In particular, the result showed that MT concentrations in the transgenic cells on the 6th day of culturing were much lower than those in the precultured transgenic cells but remained the same in the nontransgenic cultures on the 6th day as in preculture cells. This may suggest that the cells would retain a threshold level by exporting the overproduced MT, although decreased synthesis of MT in these cells cannot be excluded.

Although the mechanism for the decrease in MT concentrations in the transgenic cardiomyocytes is unknown, the cells indeed contained 2-fold MT at the time they were exposed to DOX and showed significant resistance to the toxicity of DOX by four distinct measures. Moreover, changes in other antioxidant components were the same between the transgenic and nontransgenic cardiomyocytes. This study, together with our in vivo observations (Kang et al., 1997), thus provides direct evidence to show that MT is involved in the cardioprotection against DOX toxicity. Also, the present study demonstrates that a 2-fold increase in MT concentrations in the cardiomyocytes was high enough to be effective in this protective action.

A critical examination is required to elucidate the quantitative distribution of the elevated MT among different cell types in the heart in vivo between the transgenic mice used here and those used by others (DiSilvestro et al., 1996). MT overexpression in the heart is driven by different mechanisms between the two transgenic mouse models (Palmiter et al., 1993; Kang et al., 1997). The use of the α-cardiac myosin heavy chain promoter directs the expression of MT specifically in the cardiomyocytes in our transgenic mice. MT was indeed found in the cardiomyocytes, as demonstrated in the present study. An important comparison between our results and those of others (DiSilvestro et al., 1996) involves whether MT is present in the cardiomyocytes in vivo at an effective concentration.

Several other explanations can be proposed regarding the discrepancy between the results presented here and those published previously (DiSilvestro et al., 1996). In the latter study, the toxicity of DOX was first assessed by mortality rate by using a normally lethal dose, with no significant difference observed between the transgenic and nontransgenic mice. This end point, however, does not specifically

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**Fig. 5.** Effect of DOX on cell viability between MT-overexpressing transgenic cardiomyocytes and nontransgenic controls determined by a short-term survival (MTT) assay. Cells were cultured at a density of 25,000 cells/well for 48 h before treated with varying concentrations of DOX for 3 h. Tetrazolium reduction was measured 48 h after DOX removal. Each point is mean ± S.D. (n = 12). *p < .05 at corresponding concentrations.

**Fig. 6.** Lipid hydroperoxide concentrations in MT-overexpressing transgenic cardiomyocytes and nontransgenic controls after exposed to 2.0 μM DOX for 24 h. Values represent the mean ± S.D. (n = 5). *Significantly different from other treatments (p < .05).
reflect cardiotoxicity, which has never been shown to be correlated with mortality, particularly at the lethal dose of DOX. The same criticism can be applied to the peritoneal fluid accumulation, which is not a specific end point of cardiotoxicity.

A specific measurement for cardiac oxidative injury by DOX was performed by examining the concentrations of 4-hydroxy-2-(E)-nonenal and malonaldehyde in previous studies (DiSilvestro et al., 1996), which showed that MT transgenic mice actually displayed higher lipid peroxide concentrations in the heart in treated with DOX. This study did not offer any explanation as to why the transgenic mouse heart showed higher concentrations of lipid peroxide products by DOX treatment. However, the colorimetric assays for 4-hydroxy-2-(E)-nonenal and malonaldehyde are nonspecific. An important problem in using these byproducts as indicators of lipid peroxidation is that the byproduct formation is highly inefficient and varies according to the transition metal ion content of the sample (Esterbauer et al., 1991). Because MT binds with metals and the composition of transition metal ions may be altered in the MT-overexpressing transgenic heart, the measurement thus may be interfered by the presence of high concentrations of this protein.

In the present study, the extent of lipid peroxidation was estimated by lipid hydroperoxide concentrations. This measure the hydroperoxides directly utilizing the redox reaction with ferrous ions (Mihaljevic et al., 1996). Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thioctanoyl ion as the chromogen. The assay must be performed in chloroform to circumvent the problems of ferric ions present in the sample and the hydrogen peroxide, which readily reacts with ferric ions to give an overestimation of lipid peroxidation. The result clearly showed that the lipid hydroperoxide concentrations in the MT transgenic cardiomyocytes were significantly suppressed.

In summary, using a primary neonatal cardiomyocyte culture established from a specific cardiac MT-overexpressing transgenic mouse model, we demonstrate that MT is involved in cardiac protection against DOX toxicity, as assessed by morphological alterations, cell viability, and LDH leakage from the cells. This cytoprotective effect of MT was correlated with its inhibition of DOX-induced lipid peroxidation, indicating that scavenging reactive oxygen species is at least one of the mechanisms by which MT functions in this cytoprotection. The effective concentrations of MT in these cells were 14:19–44:1, showing that scavenging reactive oxygen species is at least one mechanism by which MT functions in this cytoprotection. The effective concentrations of MT in these cells were about 2-fold higher than normal. These observations provide direct evidence to confirm the in vivo observations regarding the role of MT in protection from DOX cardiotoxicity.

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References


Send reprint requests to: Dr. Y. James Kang, Department of Medicine, University of Louisville School of Medicine, 530 S. Jackson St., Louisville, KY 40202. E-mail: yjkang01@homer.louisville.edu