Metallothionein Acts as a Cytoprotectant against Doxorubicin Toxicity

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ABSTRACT
The protective role of metallothionein (MT) against the myocardiotoxicity and hepatotoxicity of doxorubicin (Dox) was investigated in mice. Dox-induced elevations of plasma creatine kinase activity, a measure of myocardiac damage, and plasma glutamate pyruvate transaminase activity, reflecting hepatic damage, were prevented by pretreatment with an MT inducer. Pretreatment with zinc induced MT in the liver and heart, thereby reducing Dox toxicity in these two organs. Pretreatment with n-hexane also induced MT and reduced Dox toxicity, but only in the liver. In primary hepatocyte cultures, the leakage of lactate dehydrogenase induced by Dox was prevented by zinc pretreatment. These results suggest that MT induction prevents Dox toxicity in vivo and in vitro. Furthermore, we determined that MT-null mice were more sensitive to the myocardiotoxic and hepatotoxic effects of Dox. These findings indicate that both basal and induced MT protect against Dox toxicity.

Doxorubicin (Dox), an anthracycline anticancer drug, is widely used against variety of human tumors. However, clinical use of Dox in a sufficient dose is limited because of its myocardiotoxicity. It has been reported that preinduction of myocardial metallothionein (MT) production by administration of zinc or bismuth protects myocardial cells from Dox toxicity (Satoh et al., 1988). MT is a cysteine-rich metal-binding protein and is induced by various metals, glucocorticoids, and other factors. MT is thought to be involved in homeostasis of essential metals and in the resistance to heavy metals such as cadmium (Hamr, 1986). It also has been reported that MT plays an important role in protection against the toxic effects of anticancer drugs (Kondo et al., 1995; Nakagawa et al., 1995) and in multiple drug resistance (Saika et al., 1994; Satoh et al., 1994; Okazaki et al., 1998). The action of MT seems to be based on its ability to scavenge hydroxy radicals (Sato and Bremner, 1993). MT inducers produce effects other than MT induction. Therefore, the participation of MT in protection against Dox toxicity is still unclear. Dox also has been shown to produce hepatotoxicity (Ganey et al., 1988). We hypothesized that if MT acts as a cytoprotectant against Dox, hepatic MT also would prevent the hepatotoxicity of Dox. The pretreatment with zinc induces not only cardiac but also hepatic MT, whereas inflammatory agents, such as n-hexane (HX), induce MT in only the liver. Thus, we compared the protective effect of zinc pretreatment to that of HX.

Recently, MT-deficient (MT-null) mice were generated by homologous recombination of MT-I and II genes (Michalska and Choo, 1993; Masters et al., 1994). With these mice, the roles of MT in the detoxication of heavy metals (Michalska and Choo, 1993; Masters et al., 1994; Satoh et al., 1997b), cisplatin (Satoh et al., 1997a), and paracetamol (Roche et al., 1998) were clarified. However, Itoh et al. (1997) and Liu et al. (1998b) previously reported that MT inducers, such as zinc, protect the liver from the toxic effects of carbon tetrachloride by an MT-independent mechanism. The MT-null mice are useful to determine the biological and toxicological roles of MT.

We designed the present study to clarify the role of basal and induced MT on the detoxication of Dox. Our results demonstrate that zinc and HX protect only MT-induced tissue from Dox toxicity, and MT-null mice were more sensitive to Dox toxicity. The results indicate that basal and induced MT act as cytoprotectants against Dox toxicity.

Materials and Methods

Reagents. Dox was kindly provided by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). 109CdCl2 was purchased from New England Nuclear (Boston, MA).

ABBREVIATIONS: Dox, doxorubicin; MT, metallothionein; HX, n-hexane; CK, creatine kinase; GPT, glutamate pyruvate transaminase; LDH, lactate dehydrogenase.
**Animals.** Six-week-old male ICR mice were supplied by Clea Japan (Osaka, Japan). MT-null mice and their corresponding controls (129/Sv) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as a closed colony in our laboratory. All mice were housed under conditions of controlled temperature (23–24°C) and light (12-h light/dark cycle). Food and tap water were provided ad libitum.

**Animal Experiments.** To determine the myocardialprotective and hepatoprotective activity of zinc, mice were administered zinc (300 μmol/kg s.c.) once a day for 2 days. These mice were given a single dose of Dox (35 μmol/kg i.p.) 24 h after the final administration of zinc. Four days after Dox administration, blood was collected. To evaluate myocardialprotective activity, heart damage was estimated by measuring creatine kinase (CK) activity in the plasma. Hepatic damage, measured by the activity of glutamate pyruvate transaminase (GPT) in the plasma, was used to evaluate the hepatoprotective activity of zinc. Plasma CK and GPT were determined by the rate assay (Morgans and Robert, 1983; Wilkie, 1983). To determine myocardialprotective and hepatoprotective activity of HX, mice were given a single dose of HX (35 μmol/kg i.p.) 48 h after administration of HX (76.6 μmol/kg s.c.). The experimental procedures for HX were same as described for zinc administration.

To determine myocardial and hepatic MT induction by zinc, mice were s.c. injected with zinc (300 μmol/kg) once a day for 2 days. Twenty-four hours after the last administration, mice were sacrificed by decapitation and the heart and liver were excised. To determine myocardial and hepatic MT induction by HX, mice were administered HX (76.6 μmol/kg s.c.). Forty-eight hours after administration, mice were sacrificed by decapitation and the heart and liver were excised. The concentration of MT in the heart and liver was determined by 109Cd/hemoglobin affinity assay (Eaton and Toal, 1982).

**Primary Culture of Hepatocytes and Treatment of Cells.** Hepatocytes were isolated by the in situ two-step collagenase perfusion method of Seglen as modified by Klaunig et al. (1981). The cells showing viability in excess of 80% as estimated by trypan blue exclusion test were used in the culture experiments. Cells were plated in Williams’ medium E containing 5% fetal calf serum onto a 12-well culture plate precoated with collagen at a density of 3.6 × 10^5 cells/well. Two hours after incubation at 37°C in 5% CO2-95% air, the medium was changed to remove unattached cells. Cells were precultured for 24 h, then treated with zinc (100 μM) for 24 h. Zinc pretreated cells were then treated for 48 h with indicated concentrations of Dox. The activity of lactate dehydrogenase (LDH) in the culture supernatant was determined by the rate assay (Morgans and Robert, 1983; Willie, 1983). To determine the effect of zinc on the MT concentration in hepatocytes, cells were treated with zinc (100 μM) for 24 h. The cells were rinsed with cold PBS and centrifuged at 2000g for 10 min. The pellet was homogenized with 10 mM Tris-HCl (pH 8.0). After centrifugation at 10,000g for 15 min, the concentration of MT in the supernatant was determined by 109Cd/hemoglobin affinity assay.

**Statistical Analysis.** The data were analyzed by ANOVA and Fisher's protected least-significant difference test. Differences between groups were considered significant at P < .05.

**Results**

The effects of zinc and HX on Dox toxicity and MT level were examined. Mice were administered zinc (300 μmol/kg) by two s.c. injections 24 h apart. Twenty-four hours after the second injection, the heart and liver were removed one group of mice and MT concentration in these tissues was determined. The other mice were administered Dox 35 μmol/kg i.p. and blood was collected 4 days after Dox administration to determine plasma CK and GPT activities. Myocardotoxicity and hepatotoxicity of Dox are highly correlated with plasma CK and GPT activity, respectively. Both toxicities occurred in the same range of Dox concentrations. Pretreatment with zinc suppressed plasma CK and GPT elevation (Fig. 1A) and induced myocardial and hepatic MT 1.8- and 21-fold, respectively (Fig. 2A). The liver-specific MT inducer HX (76.6 μmol/kg s.c.) increased hepatic MT (18-fold) 48 h after its administration. HX inhibited the Dox-induced plasma GPT elevation (Fig. 1B), but did not affect Dox-induced plasma CK elevation.

The effect of zinc on Dox toxicity was examined in primary cultured hepatocytes. Dox toxicity in primary hepatocytes was evaluated by LDH leakage from cells. Dox-induced LDH leakage appeared 48 h after treatment and was prevented by a 24-h pretreatment with 100 μM zinc (Fig. 3). Twenty-four hours after zinc treatment, MT was induced 3-fold (data not shown).

MT-null mice were sensitive to the myocardotoxic and hepatotoxic effects of Dox. Dox-induced elevation of plasma CK and GPT activities in wild-type mice, containing hepatic and myocardial MT, were 8.9- and 1.6-fold, respectively. In MT-null mice, Dox treatment elevated plasma CK and GPT activity to 12.1- and 2.8-fold, respectively (Fig. 4). The elevation of plasma CK and GPT activities in MT-null mice were significantly different from wild-type mice.

**Discussion**

Many investigators reported that MT, due to its free radical-scavenging capability, may play an important role in reducing the toxic side effects of various anticancer drugs. But, it is not clear that MT acts as cytoprotectant against Dox cardiotoxicity. Dox produces both cardiotoxicity, a complicating factor in Dox chemotherapy, and hepatotoxicity. The present study demonstrates that induced and basal MT protect against Dox myocardotoxicity and hepatotoxicity. There are several hypotheses to explain Dox-induced myocardotoxicity ( Olson and Mushlin, 1990). Among them, the free radical hypothesis is the most thoroughly investigated (Lee et al., 1991). Dox undergoes one-electron reduction through a metabolic activation caused by NADPH-cytochrome P-450 reductase or other flavin-containing enzymes in microsomes ( Bachur et al., 1978). This reduction generates...
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In the presence of molecular oxygen, the semiquinone rapidly reduces oxygen to superoxide, and the intact Dox remains. Superoxide spontaneously converts to hydrogen peroxide or is rapidly converted by superoxide dismutase. This hypothesis is supported by a study with transgenic mice overexpressing the catalase gene in the heart (Kang et al., 1996). The physiological function of catalase is to detoxify hydrogen peroxide, and the mice in the study by Kang et al. (1996) were resistant against Dox toxicity.

However, the Dox semiquinone can react with hydrogen peroxide to yield hydroxyl radicals (Kalyanaraman et al., 1984). These highly toxic reactive species may be scavenged by MT (Thorunolley and Vasak, 1985; Quesada et al., 1996). Satoh et al. (1988) reported that the markedly reduced myocardial toxicity of Dox by pretreatment of bismuth or zinc, which act as MT inducers, is due to induced myocardial MT. Recently, to clarify whether MT provides protection from Dox-induced myocardial toxicity, two groups studied mice overexpressing MT. One report found that MT transgenic mice were not resistant to the cardiotoxicity of Dox (DiSilvestro et al., 1996). However, in the other report, MT acted as a cytoprotectant against cardiotoxicity (Kang et al., 1997). The contradictory findings of these reports may arise from the use of different types of transgenic mice. Myocardial MT levels in the transgenic mice used by DiSilvestro et al. (1996) and Kang et al. (1997) were 3- and 10- to 130-fold versus control mice, respectively. These results suggest that higher levels of MT may protect against Dox toxicity. We hypothesized that hepatic MT would prevent Dox hepatotoxicity if MT acts as radical scavenger against Dox-generated hydroxyl radicals. Cisplatin causes severe nephrotoxicity and has an affinity for MT (Pattanaik et al., 1992; Lemkuil et al., 1994). In cisplatin toxicity, it is reported that MT protects against not only cisplatin-induced nephrotoxicity but also hepatotoxicity (Liu et al., 1998a). The present article investigated the ability of MT to act as radical scavenger against Dox in both the heart and liver.

In mice receiving toxic dose of Dox, preadministration of zinc induces not only myocardial protection but also hepato- protection. Dox hepatotoxicity also was protected by HX (Fig. 1), although it showed no effect against Dox myocardial toxicity. Zinc and HX are well known MT inducers that induce MT by different mechanisms. Zinc directly induces MT in various tissues (Westin and Schaffner, 1988), whereas HX, mediated by an acute-phase response, indirectly induces MT in only the liver (Min et al., 1991). DiSilverstro and Joseph (1995) reported that an acute-phase response does not elevate heart MT levels in rats, nor does it inhibit the cardiotoxicity of Dox. These results indicates that MT induced tissues were resistant to Dox toxicity. On the basis of the free radical hypothesis presented in the above-mentioned articles, the MT-related protection observed in our study may be due to its radical scavenging capability.

Dox toxicity is observed in primary myocytes and hepatocytes and is mediated by free radicals. In primary hepatocytes, MT is induced by zinc. If MT acts as cytoprotectant against Dox toxicity, the protective effect of zinc must be observed in primary hepatocytes. Because zinc pretreatment did reduce cytotoxicity (Fig. 3), the in vivo and in vitro hepatocytes studies indicate that induced MT reduces the toxic effects of Dox.

From these studies, our results suggest that induced MT acts as a protectant against Dox toxicity. However, several MT inducers produce not only MT induction but also increase hepatic glutathione (GSH) levels (Izard et al., 1995). It is thought that GSH acts as a guard against Dox toxicity (Doroshow et al., 1981; Yoda et al., 1986; Villani et al., 1990).
Although the peroxide radical-scavenging activity of MT is ~100 times greater than GSH on a molar basis (Miura et al., 1997), protection might be due to the MT-independent effects of the MT inducer, such as an elevated GSH level. However, our study shows that Dox is more toxic in both the heart and liver of MT-null mice than in wild-type mice (Fig. 4). These results indicated that basal MT protects against the toxic effect caused by Dox.

In conclusion, induced and basal MT protect against Dox toxicity, and both zinc- and HX-induced protection appeared to be due to the induction of MT. Furthermore, these results support the free radical hypothesis of Dox toxicity. More importantly, Naganuma et al. (1988) reported that pretreatment of bismuth substrate did not affect the anticancer activity in tumor-bearing mice, although its myocardotoxicity was significantly depressed. Pretreatment of bismuth substrate induced cardiac MT but did not affect tumor MT. Therefore, tissue-specific MT induction in the heart has potential for clinical applications in chemotherapy. We designed the study to clarify the protective role of MT against acute Dox toxicity. Dox toxicity was reviewed from a clinical perspective (Shan et al., 1996). Chronic Dox toxicity is more important than the acute toxicity. However, there was no information about the interaction of MT and its inducers on the prevention of chronic toxicity. Experiments that investigate the ability of MT to protect against chronic toxicity will be of great interest.

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