Recurrence of Toxicity by Cadmium Released from Accumulated Cadmium-Metallothionein in Mice

Kyong-Son Min,* Kayo Shida, Naoko Tanaka, Nozomu Yamashita, Noriko Tetsuchikawahara, and Satomi Onosaka

Faculty of Nutrition, Kobe Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan

(Received February 10, 2005; Accepted February 19, 2005; Published online February 25, 2005)

Cadmium (Cd) is a widespread toxic pollutant that enters humans and animals through the food chain. Cd usually is accumulated by binding to metallothionein (MT), which appears to be responsible for its detoxification in the cell. To investigate whether the Cd released from Cd-MT can cause toxicity to recur, we studied the effects of oxidative stress and contamination with multiple metals on the release of Cd from accumulated Cd-MT and recurrence of toxicity *in vitro* and *in vivo*. Incubation of Cd-MT with H_2O_2, ferric nitrilotriacetate (Fe-NTA) and H_2O_2, or Cu^{2+} resulted in release of Cd from its binding with MT. *In vivo* study, Cd was released from renal Cd-MT after mice that had accumulated Cd-MT were injected with Fe-NTA. Addition of purified Cd-MT to mouse liver cytosol did not result in inhibition of cytosolic superoxide dismutase (SOD) activity. However, treatment of Cd-MT with H_2O_2 or Cu^{2+} led to the release of Cd^{2+} from Cd-MT, which inhibited cytosolic SOD activity. Simultaneous injection with Cu^{2+} and a non-acute toxic dose of Cd significantly increased plasma aminotransferase activities, indicating hepatic injury, in mice that had accumulated Cd-MT but not in those that had accumulated Zn-MT. The hepatic concentration of Cu increased with the injected dose and the concentration of Cd in the MT fraction decreased. These results suggest that contamination with metals whose affinities for MT are higher than that of Cd may cause recurrent toxicity due to the release of Cd from accumulated Cd-MT.

Key words —— released cadmium, accumulated cadmium-metallothionein, cadmium toxicity

*To whom correspondence should be addressed: Faculty of Nutrition, Kobe Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe 651–2180, Japan. Tel.: +81-78-974-1551; Fax: +81-78-974-5689; E-mail: min@nutr.kobegakuin.ac.jp

INTRODUCTION

Cadmium (Cd) is an environmental and industrial pollutant that poses a serious health risk to humans and animals. Cd^{2+} has multiple cytotoxic and metabolic effects, such as interfering with the normal actions of essential metals,[1] inducing oxidative stress,[2] and altering the activities of various enzymes.[3,4] After Cd exposure, Cd is distributed mainly to the liver and kidneys and accumulates with metallothionein (MT). MT is a small metal-binding protein that is characterized by its high thiol content: 20 of its 61 amino acids are cysteine, all of which are in the reduced state and are involved in its metal binding properties. MT-I transgenic mice are resistant to Cd-induced lethality and hepatotoxicity.[5] In comparison, MT-null mice show increased susceptibility to Cd-induced lethality and liver injury, and lack Cd accumulation.[6] MT may sequester Cd^{2+} from molecular targets by binding to Cd^{2+} with high affinity and thus making it less available for excretion. Therefore, with chronic exposure to Cd^{2+} at non-acute toxic doses, MT appears to accumulate Cd in a less toxic form until reaching the critical level.[7,8] Conversely, if, under certain conditions, Cd^{2+} is released from its bond with MT, Cd toxicity may appear again.

MT recently has received increased attention due to its ability to act as a scavenger of free radicals.[9,10] Hydrogen peroxide and free radicals induce loss of the metal-binding properties of MT by oxidation of its thiol residues.[11] In addition, the affinity of Cu for the metal-binding sites on MT is substantially greater than that of Zn or Cd.[12] Therefore, we studied here *in vitro* and *in vivo* whether Cd^{2+} could be released from its binding to MT after oxidative stress and treatment with Cu^{2+} to become toxic again.

MATERIALS AND METHODS

Animals —— Male mice (ddY strain) 6 weeks old and weighing approximately 30 g were purchased from Nihon SLC (Shizuoka, Japan). Mice were maintained on a 12 : 12-hr light : dark cycle and given food and water *ad libidum* (Type MF, Oriental Yeast, Osaka, Japan). All animal experiments were done under the control of the Animal Research Committee, in accordance with the Guidelines on Animal Experiments of Kobe Gakuin University and the Japanese Government Animal Protection and Management Law (No. 105).

Chemicals —— All chemicals were purchased from Nakarai Tesque, Inc. (Kyoto, Japan). The kits for measuring aminotransferase activities were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Release of Cd from Cd-MT *in Vitro* —— Cd-MT was purified from the livers of Cd-treated rats (1 mg Cd/kg daily for 5 days) according to a previous method.[12] Purified Cd-MT contained only Cd, not Zn or Cu. Purified Cd-MT (3.2 μg) was incubated with 10 mM H_2O_2, with ferric nitriloacetate (Fe-NTA; 50 μM) and H_2O_2 (2.8 mM), or with Cu^{2+} (0.9 μg) in 10 mM Tris–HCl (pH 7.4). After incubation, all reaction mixtures were applied to a Sephadex G-75 column (1.5 × 28 cm) equilibrated with 10 mM Tris–HCl (pH 8.0). The column was eluted with the same buffer, and 2-ml fractions were collected.

Release of Cd or Zn from Binding to MT after an Injection with Fe-NTA to Cd-MT or Zn-MT Accumulated Mice —— Mice were pretreated with $CdCl_2$ (1 mg Cd/kg once daily subcutaneously for 6 days) or with $ZnSO_4$ (20 mg Zn/kg once daily subcutaneously for 2 days). The aim was to accumulate Cd-MT or Zn-MT; these mice are here after referred to Cd-MT and Zn-MT accumulated mice, respectively. Two days after the last injection, Cd-MT or Zn-MT accumulated mice were injected subcutaneously with Fe-NTA (7.5 mg/kg). After 4 hr, mice were euthanized by over-anesthesia. The kidneys were obtained and homogenized with 10 mM Tris–HCl (pH 8.0). The concentration of Cd bound to MT was determined by using the Cd-hem method as described previously.[13]

Activity of Superoxide Dismutase (SOD) in Mouse Cytosol Incubated with Cd Released from Cd-MT —— Cytosol was prepared from intact mouse livers. Incubation mixtures of purified Cd-MT (100 μg as Cd) with 10 mM H_2O_2 or Cu^{2+} (30 μg) were concentrated by lyophilization to remove excess H_2O_2 and then were filtered by Ultrafree-MC 5000 (Millipore Co., Billerica, MA, U.S.A.) to remove MT. These filtrates contained only Cd^{2+}, and not Cu^{2+}. After either purified Cd-MT or these filtrates was added to mouse cytosol, the SOD activity in each of these cytosol was measured using the inhibition of nitroblue tetrazolium (NBT) reduction due to superoxide anion generation by xanthine plus xanthine oxidase (NBT method).[14]

Simultaneous Injection with Cu^{2+} and Cd^{2+} to Cd-MT or Zn-MT Accumulated Mice —— Seven days (Cd-MT accumulated mice; 1 mg/kg daily for 6 days) or 1 day (Zn-MT accumulated mice; 20 mg/kg daily for 6 days) after the last injection with Cd or Zn to mice, various doses of Cu^{2+} (0–2 mg/kg) were injected intravenously with or without $CdCl_2$ (0.8 mg Cd/kg). After 4 or 24 hr, mice were euthanized by over-anesthesia. Heparinized blood was collected. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were determined spectrophotometrically by using Transaminase CII-Test kits (Wako Pure Chemical Industries, Ltd.). After sections of liver was homogenized with 0.25 M sucrose, the tissue homogenates were digested with nitric acid overnight, and the concentrations of Cd and Cu were analyzed using flame atomic absorption spectrophotometry (AAS; Z-5000 Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Tokyo, Japan). Cytosol prepared from the homogenates of liver after 4 hr was applied to Sephadex G-75 columns under the same conditions as for the *in vitro* study.

Statistical Analysis —— Results are expressed as mean ± 1 standard deviation (S.D.). Data were analyzed for significance by using Student's *t*-test and by a two-tailed analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

RESULTS

Release of Cd from Binding to MT

Figure 1 shows the gel filtration profiles of Cd-MT after its incubation with H_2O_2, Fe-NTA and H_2O_2 or Cu^{2+}. Incubation of Cd-MT with H_2O_2 (Fig. 1A), with Fe-NTA and H_2O_2 (Fig. 1B), or with Cu^{2+} (Fig. 1C) resulted in a decrease of Cd concentration in MT fraction and an increase of Cd concentration in low-molecular-weight (LM) fraction. Further, injection with Fe-NTA caused a decrease in the concentration of Zn or Cd bound to renal MT in Zn-MT

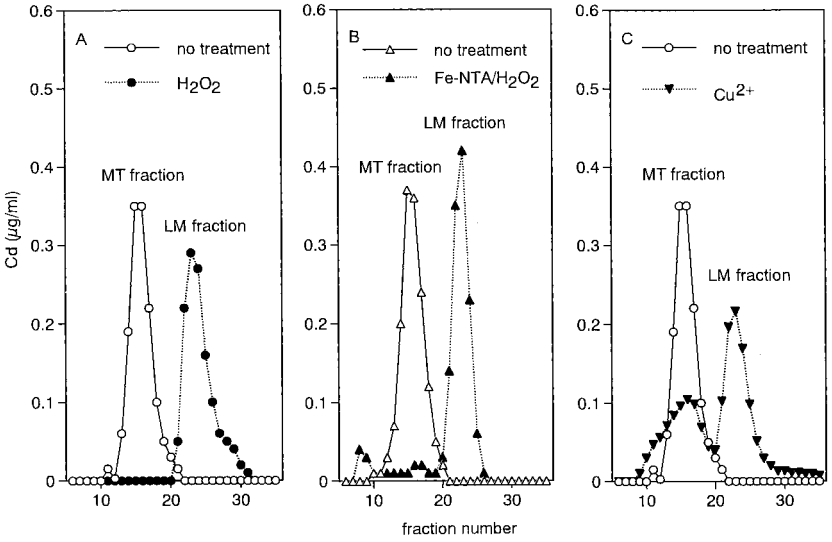

Fig. 1. Release of Cd^{2+} from Cd-MT after Incubation of Cd-MT with H_2O_2, Ferric Fe-NTA and H_2O_2, or Cu^{2+}

After Incubation of purified Cd-MT with H_2O_2 (A), Fe-NTA and H_2O_2 (B), or Cu^{2+} (C) in 10 mM Tris–HCl (pH 7.4), all reaction mixtures were applied to a Sephadex G-75 column. The concentration of Cd in each fraction was determined by AAS. MT fraction, metallothionein fraction; LM fraction, Low-molecular-weight fraction.

or Cd-MT accumulated mice, respectively (Fig. 2). These data indicate that reactive oxygen species and some metals whose affinity for MT is higher than that of Cd can cause the release of Cd^{2+} from Cd-MT *in vivo*.

In Vitro Inhibition of Cytosolic SOD Activity by Cd Released from Cd-MT

Figure 3 shows the activity of cytosolic SOD after incubation of Cd-MT with H_2O_2 or Cu^{2+} *in vitro*. Addition of Cd-MT to cytosol did not inhibit cytosolic SOD activity. However, treatment of Cd-MT with H_2O_2 or Cu^{2+} led to the release of Cd, which in turn increasingly inhibited cytosolic SOD activity as the concentration of Cd increased. These results suggest that Cd^{2+} released from Cd-MT after oxidative stress or by replacement with Cu^{2+} can cause inhibition of cytosolic SOD activity.

Simultaneous Injection of Cd-MT Accumulated Mice with Cu^{2+} and Cd^{2+}

Figure 4 shows the plasma activities of ALT (A) and AST (B) after simultaneous injection with Cu^{2+} (0–2 mg/kg) and Cd^{2+} (0.8 mg/kg) to Cd-MT accumulated mice. Though an injection with Cu^{2+} or Cd^{2+} alone did not elevate the plasma activities of ALT and AST, simultaneous injection with Cu^{2+} at a higher dose than 0.75 mg/kg and Cd^{2+} resulted in a marked increase in both enzyme activities, indicating liver injury. Figure 5A shows the hepatic concentrations

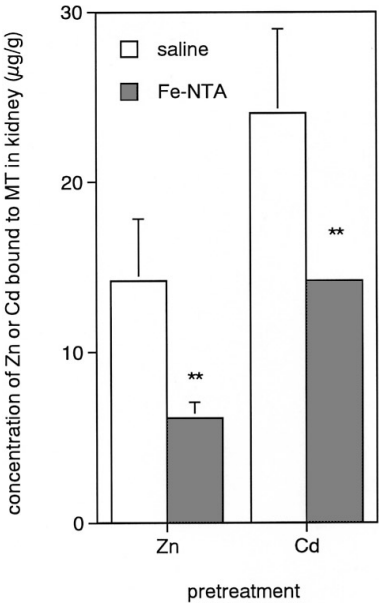

Fig. 2. Release of Zn or Cd from Accumulated Zn-MT or Cd-MT after an Injection with Fe-NTA to Zn-MT or Cd-MT Accumulated Mice

Either Cd-MT (1 mg/kg daily for 6 days) or Zn-MT (20 mg/kg daily for 2 days) accumulated mice were injected with saline or Fe-NTA (7.5 mg Fe/kg); 4 hr later, the kidneys were obtained and the renal Cd and Zn concentrations were determined. Data points represent mean ± S.D. ($n = 4$). **, $p < 0.01$ *versus* saline mice.

of Cd and Cu after simultaneous injection with Cd and various doses of Cu^{2+}. The hepatic concentration of Cu, but not Cd, increased with increasing

Cu^{2+} dose. Simultaneous injection with Cu^{2+} and Cd^{2+} to Cd-MT accumulated mice caused a remarkable decrease in MT fraction (Fig. 5B). These findings indicate that binding of Cu^{2+} to hepatic MT caused the release of Cd from accumulated Cd-MT. In contrast, simultaneous injection of Zn-MT mice with Cu^{2+} and Cd^{2+} did not induce hepatic injury (Fig. 6). These results suggest that Cd released from accumulated Cd-MT by simultaneous injection of Cd-MT accumulated mice with Cu^{2+} and Cd^{2+} may participate in development of hepatic injury.

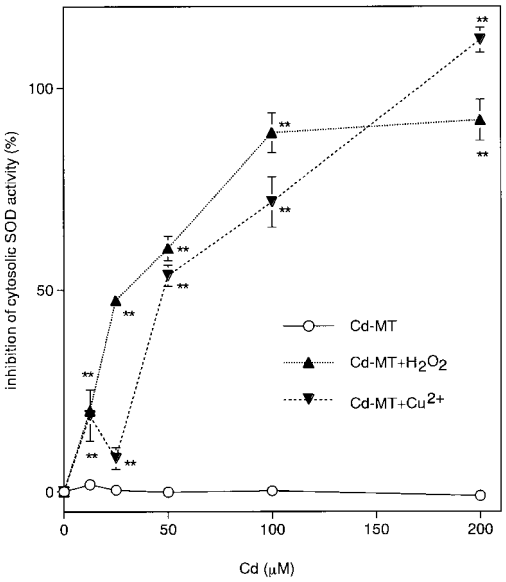

Fig. 3. Inhibition of Cytosolic SOD Activity by Cd Released from Cd-MT

Incubation mixtures of purified Cd-MT with H_2O_2 ("point up" triangles) or Cu^{2+} ("point down" triangles) were filtered to remove MT. After mouse cytosol was incubated with purified Cd-MT (open circles) or these filtrates, the SOD activity in each incubation mixture was measured using NBT method. Data points represent mean ± S.D. (n = 4). **, $p < 0.01$ *versus* 0 μM Cd.

DISCUSSION

The Cd accumulated in target organs (*e.g.*, liver, kidney) is not toxic until it reaches each critical Cd concentration after chronic Cd exposure.[7,8] Most of the Cd is accumulated with MT in the cell; therefore, MT may sequester Cd^{2+} from molecular targets by binding to Cd^{2+} with high affinity. Even if treatment with a hepatoxin or nephrotoxin induces the redistribution of Cd accumulated in the liver and kidney to the plasma, the Cd is transferred along with MT.[15] However, we demonstrated here *in vivo* that Fe-NTA induced the release of not only Zn from its binding to MT, but also MT-bound Cd. Maret suggests that release of the biologically redox-insert Zn^{2+} follows reaction of the redox-active cysteine sulfur ligand with an oxidant.[16] Whereas Zn^{2+} plays essential roles in almost all aspects of metabolism, the Cd released from Cd-MT may appear to toxic action again. Our *in vitro* study shows that the Cd released from Cd-MT inhibited the cytosolic

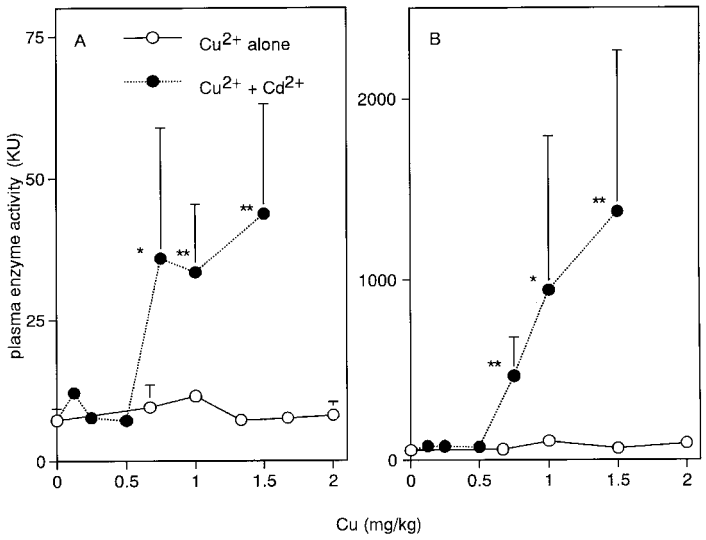

Fig. 4. Activity of Plasma Aminotransferases after Simultanious Injection with Cu^{2+} and Cd^{2+} to Cd-MT Accumulated Mice

An injection with Cu^{2+} alone (opened circles) or simultaneous injection with Cu^{2+} and Cd^{2+} (0.8 mg/kg) (filled circles) to Cd-MT accumulated mice (1 mg/kg daily for 6 days). Plasma was obtained 24 hr after the injection, and the plasma activities of ALT (A) and AST (B) were determined. Data points represent mean ± S.D. (n = 4). *, $p < 0.05$ *versus* Cu^{2+} alone; **, $p < 0.01$ *versus* Cu^{2+} alone.

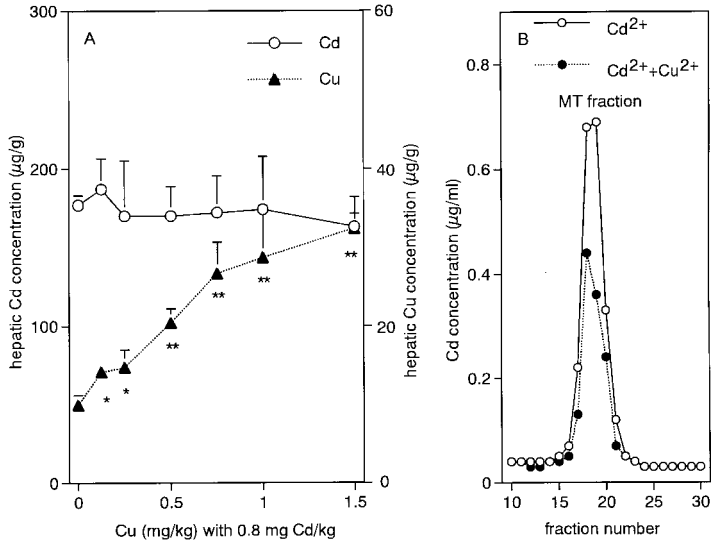

Fig. 5. Hepatic Cu uptake (A) and Gel-Filtration Profile of Hepatic Cytosol (B) after Simultaneous Injection with Cu^{2+} and Cd^{2+} to Cd-MT Accumulated Mice

(A) At 24 hr after injection, the liver was obtained and digested with nitric acid. The Cu (triangles) and Cd (circles) concentrations were analyzed using AAS. (B) At 4 hr after an injection with Cd (0.8 mg/kg) alone (open circles) or simultaneous injection with Cd (0.8 mg/kg) and Cu (1 mg/kg; filled circles), each cytosol prepared from the liver was applied to a Sephadex G-75 column as for Fig. 1. Data points represent mean ± S.D. (n = 4). *, $p < 0.05$ *versus* Cd alone; **, $p < 0.01$ *versus* Cd alone.

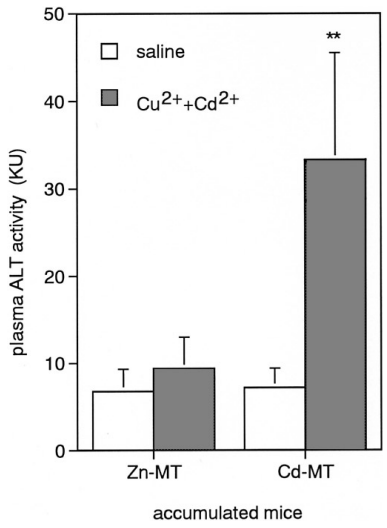

Fig. 6. Plasma Alanine Aminotransferase (ALT) Activity after Simultaneous Injection with Cu^{2+} and Cd^{2+} to Zn-MT or Cd-MT Accumulated Mice

The ALT activity in the plasma was determined 24 hr after simultaneous injection with Cu^{2+} (1 mg/kg) and Cd^{2+} (0.8 mg/kg) or an injection with saline to Zn-MT (20 mg/kg daily for 6 days) or Cd-MT (1 mg/kg daily for 6 days) accumulated mice. Data points represent mean ± S.D. (n = 4). *, $p < 0.05$ *versus* saline.

SOD activity with increased its concentration. However, it is difficult to investigate the recurrence of toxicity due to the Cd released from Cd-MT because of the antioxidant functions of MT under oxidative stress *in vivo*.[17]

It is well known that Cu and Hg have higher affinity for MT than do Cd and Zn.[12] In the present data, addition of Cu^{2+} to Cd-MT led to release of Cd from MT *in vitro*. However, injection of Cd-MT accumulated mice with various doses of Cu^{2+} did not induce hepatotoxicity. Because accumulated MT contained not only Cd but also Zn in Cd-MT accumulated mice, the amount of Cd released from Cd-MT may not have been enough to induce hepatotoxicity. Interestingly, simultaneous injection with Cu^{2+} and Cd^{2+} caused hepatic injury in Cd-MT accumulated mice but not Zn-MT mice, whereas injection with a similar dose of Cu^{2+} or Cd^{2+} alone failed to induce hepatotoxicity. Moreover, simultaneous injection with Cu^{2+} and Cd^{2+} led to a decrease in the concentration of Cd in hepatic MT fraction of Cd-MT accumulated mice. These data suggest that the Cd released from accumulated Cd-MT may be concerned to initiate hepatotoxicity after simultaneous injection with Cu^{2+} and Cd^{2+} to Cd-MT accumulated mice.

Although many reports suggest that Cu-MT is a prooxidant,[18] overexpression of MT in cells after Cd pretreatment, or ectopic overexpression by gene transfer, confers protection from Cu-dependent lipid oxidation and cytotoxicity. Conversely, when MT is depleted (*e.g.*, by actinomycin D), the concentration

of non-MT-associated, 'free' cytosolic Cu^{2+} is elevated, and hepatoma tissue culture cells rapidly lose their resistance to Cu toxicity, as reflected in loss of cell viability.[19] Therefore, binding of Cu to MT appears to render Cu redox-inactive, but oxidation of free thiols critical for metal binding can reduce MT/Cu interactions and potentiate Cu redox cycling.[18] If Cu is released from accumulated MT in Cd-MT accumulated mice, this free Cu in turn might enhance the release of Cd from its binding to MT.

We propose that oxidative stress and contamination with metals whose affinity for MT is higher than that of Cd may cause toxicity due to the release of Cd from its binding to MT by thiol oxidation and metal replacement. Even if the amount of Cd accumulated in human tissues is lower than the critical level,[20] the actual concentration of accumulated Cd may be a risk factor for Cd toxicity under some conditions.

Acknowledgements This study was supported by Scientific Research Grant C-11680560 from the Japanese Ministry of Education, Science, and Culture.

REFERENCES

1) Chmielnicka, J. and Sowa, B. (1996) Cadmium interaction with essential metals (Zn, Cu, Fe) metabolism, metallothionein, and ceruloplasmin in pregnant rats and fetuses. *Ecotoxicol. Environ. Saf.*, **35**, 277–281.
2) Casalino, E., Sblano, C. and Landriscina, C. (1997) Enzyme activity alteration by cadmium administration to rats: the possibility of iron involvement in lipid peroxidation. *Arch. Biochem. Biophys.*, **346**, 171–179.
3) Bagchi, D., Joshi, S. S., Bagchi, M., Balmoori, J., Benner, E. J., Kuszynski, C. A. and Stohs, S. J. (2000) Cadmium- and chromium-induced oxidative stress, DNA damage, and apoptotic cell death in cultured human chronic myelogenous leukemic K562 cells, promyelocytic leukemic HL-60 cells, and normal human peripheral blood mononuclear cells. *J. Biochem. Mol. Toxicol.*, **14**, 33–41.
4) Patra, R. C., Swarup, D. and Senapati, S. K. (1999) Effects of cadmium on lipid peroxides and superoxide dismutase in hepatic, renal and testicular tissue of rats. *Vet. Hum. Toxicol.*, **41**, 65–67.
5) Liu, Y., Liu, J., Iszard, M. B., Andrews, G. K., Palmiter, R. D. and Klaassen, C. D. (1995) Transgenic mice that overexpress metallothionein-I are protected from cadmium lethality and hepatotoxicity. *Toxicol. Appl. Pharmacol.*, **135**, 222–228.
6) Masters, B. A., Kelly, E. J., Quaife, C. J., Brinster, R. L. and Palmiter, R. D. (1994) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 584–588.
7) Suzuki, Y. (1980) Cadmium metabolism and toxicity in rats after long-term subcutaneous administration. *J. Toxicol. Environ. Health*, **6**, 469–482.
8) Min, K. S., Onosaka, S. and Tanaka, K. (1996) Renal accumulation of cadmium and nephropathy following long-term administration of cadmium-metallothionein. *Toxicol. Appl. Pharmacol.*, **141**, 102–109.
9) Sato, M. and Bremner, I. (1993) Oxygen free radicals and metallothionein. *Free Radic. Biol. Med.*, **14**, 325–337.
10) Min, K. S., Nishida, K. and Onosaka, S. (1999) Protective effect of metallothionein to ras DNA damage induced by hydrogen peroxide and ferric ion-nitrilotriacetic acid. *Chem. Biol. Interact*, **122**, 137–152.
11) Jimenez, I., Gotteland, M., Zarzuelo, A., Uauy, R. and Speisky, H. (1997) Loss of the metal binding properties of metallothionein induced by hydrogen peroxide and free radicals. *Toxicology*, **120**, 37–46.
12) Funk, A. E., Day, F. A. and Brady, F. O. (1987) Displacement of zinc and copper from copper-induced metallothionein by cadmium and by mercury: in vivo and ex vivo studies. *Comp. Biochem. Physiol. C*, **86**, 1–6.
13) Onosaka, S. and Cherian, M. G. (1982) Comparison of metallothionein determination by polarographic and cadmium-saturation methods. *Toxicol. Appl. Pharmacol.*, **63**, 270–274.
14) Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.*, **44**, 276–287.
15) Tanaka, K. (1982) Effects of hepatic disorder on the fate of cadmium in rats. In *Biological roles of metallothionein* (Foulkes, E. C., ed.), Elsevier, Amsterdam, pp. 237–249.
16) Maret, W. (2000) The function of zinc metallothionein: a link between cellular zinc and redox state. *J. Nutr.*, **130**, 1455S–1458S.
17) Min, K. S., Morishita, F., Tetsuchikawahara, N. and Onosaka, S. (2005) Induction of hepatic and renal metallothionein synthesis by ferric nitrilotriacetate in mice: the role of MT as an antioxidant. *Toxicol. Appl. Pharmacol.*, **204**, 9–17.
18) Fabisiak, J. P., Pearce, L. L., Borisenko, G. G.,

Tyhurina, Y. Y., Tyurin, V. A., Razzack, J., Lazo, J. S., Pitt, B. R. and Kagan, V. E. (1999) Bifunctional anti/prooxidant potential of metallothionein: redox signaling of copper binding and release. *Antioxid. Redox Signal*, **1**, 349–364.

19) Steinebach, O. M. and Wolterbeek, H. T. (1994) Role of cytosolic copper, metallothionein and glutathione in copper toxicity in rat hepatoma tissue culture cells. *Toxicology*, **92**, 75–90.

20) Angerer, P., Kessel, R., Bencze, K., Tewordt, M., Mauermayer, R. and Friesen, A. (1988) The cadmium content of human tissues from biopsies. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [B]*, **187**, 18–30.