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Original Article

Sensitivity of MT-III null mice upon chronic exposure to cadmium

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ABSTRACT — Cadmium (Cd) is an environmental contaminant known to exert toxic effects on various tissues. Metallothionein (MT) acts as a protective protein with high affinity for Cd. However, among the four isoforms of MT, the physiologic function of MT-III in the liver of mice exposed to Cd chronically has not been determined. In the present study, we examined the susceptibility of MT-III null mice to hepatotoxicity by exposure to Cd for 67 weeks. Cd exposure reduced the body weight of wild-type mice but not MT-III null mice. MT-I/II null mice were also exposed to Cd; as expected, they died at 18 weeks of exposure. Long-term exposure to Cd exhibited mild hepatotoxicity to wild-type mice, and the effects of MT-III on hepatotoxicity were not extensive. Long-term exposure to Cd increased mRNA levels of MT-I and MT-II in the livers of wild-type mice and MT-III null mice. These results suggest that long-term exposure to Cd may contribute similar sensitivity to the livers of MT-III null mice as that of wild-type mice because expression of MT-I and MT-II was induced in the liver of both types of mice.

Key words: Cadmium, Chronic exposure, Hepatotoxicity, Metallothionein

INTRODUCTION

Cadmium (Cd) is an environmental contaminant known to exert toxic effects on various tissues (Nordberg *et al.*, 2007). In particular, the liver, testis, kidney, lung and bone are the main target tissues of Cd toxicity (Järup *et al.*, 1998; Satoh *et al.*, 2002). In addition, Cd causes acute and chronic toxicity in the liver.

Metallothionein (MT) is a cysteine-rich low-molecular-weight protein with high affinity for various metals, such as Cd and mercury (Klaassen *et al.*, 1999). In mammals, MT has been identified in four isoforms: MT-I and MT-II are expressed in almost all tissues; MT-III is expressed mainly in the brain; MT-IV is expressed in stratified squamous epithelia (Vašák and Meloni, 2011). MT-I and MT-II act as biologic protective factors against Cd toxicity. MT-I/II null mice with disrupted genes of MT-I and MT-II have been shown to exhibit high sensitivity to Cd toxicity, such as hepatotoxicity, nephrotoxicity, and bone toxicity (Masters *et al.*, 1994; Liu *et al.*, 1996, 1998, 2000; Habeebu *et al.*, 2000a, 2000b; Honda *et al.*, 2010a). In particular, MT-I/II null mice are highly sensitive to Cd-induced acute and chronic hepatotoxicity (Masters *et al.*, 1994; Liu *et al.*, 1996; Habeebu *et al.*, 2000a; Honda *et al.*, 2010a). Our previous studies demonstrated that MT-III null mice are resistant to Cd-induced acute hepatotoxicity and testicular injury (Honda *et al.*, 2010a, 2010b). Those findings suggest, therefore, that MT-III might act as an enhancing factor in Cd-induced acute hepatotoxicity, unlike the role of MT-I and MT-II. However, the role of MT-III in Cd-induced chronic hepatotoxicity is not clear.

In the present study, we examined the susceptibility of MT-III null mice to hepatotoxicity by long-term exposure to Cd. MT-I/II null mice were also used to compare Cd-induced chronic hepatotoxicity between MT-III null mice and MT-I/II null mice.

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MATERIALS AND METHODS

Animals and treatment

All animal experiments were undertaken in accordance with the Regulations on Animal Experimentation at the School of Pharmacy, Aichi Gakuin University (Nagoya, Japan). All procedures to maintain and use mice were approved by the Animal Care and Use Committee for the School of Pharmacy, Aichi Gakuin University.

MT-III null mice, MT-I/II null mice and 129/Sv mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred routinely in the laboratory animal facility of Aichi Gakuin University. MT-III null mice and MT-I/II null mice were developed by Erickson *et al.* (1997) and Masters *et al.* (1994), respectively. MT-III null mice and MT-I/II null mice had the genetic background of the 129/Sv strain. Age- and sex-matched 129/Sv mice were used as wild-type controls. Five-week-old female mice were caged in a ventilated animal room at 23°C ± 1°C with relative humidity, and a 12-hr light-dark cycle.

Mice were assigned randomly to control or experimental groups. Control mice were fed standard laboratory chow. Experimental mice were fed chow containing 300 ppm Cd (Oriental-BioService, Kyoto, Japan). All mice had unlimited access to tap water. After 67 weeks of Cd exposure, the liver and blood were removed from each mouse under ether anesthesia.

Glutamic oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities

To evaluate hepatotoxicity, the activities of GOT and GPT in serum were examined. An automatic dry-chemistry analyzer system (Spotchem EZ SP-4430; Arkray, Kyoto, Japan) was used to determine activities.

Histopathology

The liver was fixed in 10% (v/v) neutral buffered formalin solution and embedded in paraffin. Deparaffinized serial tissue sections (thickness, 5 μm) were stained with hematoxylin and eosin.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of mouse tissue was extracted with QuickGene RNA Tissue Kit S (Fujifilm, Tokyo, Japan) according to manufacturer instructions. Total RNA was incubated with a PrimeScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa Bio, Shiga, Japan) to generate cDNA. Real-time PCR was done with SYBR Premix Ex Taq™ II (Perfect Real Time) (TaKaRa Bio) and a Thermal Cycler Dice Real-time system (TaKaRa Bio). PCR conditions were: 10 sec of hot-start at 95°C followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. Gene expression was normalized to *β-actin* mRNA levels. Oligonucleotide sequences of the primers (sense and antisense, respectively) were: 5′-TCTAAGCGTCACCACGACTTCA-3′ and 5′-GTGCACTTGCAGTTCTTGCAG-3′ for the mouse *MT-I* gene; 5′-CCTGCAATGCAAACAACAATGC-3′ and 5′-AGCTGCACTTGTCGGAAGC-3′ for the mouse *MT-II* gene; 5′-CCTAAGGCCAACCGTGAAAA-3′ and 5′-AGGCATACAGGGACAGCACA-3′ for the mouse *β-actin* gene.

Statistical analyses

Statistical analyses were undertaken using single-factor ANOVA or two-factor factorial ANOVA followed by Bonferroni's test for *post hoc* comparison ($P < 0.05$). The Student's *t*-test was used between two factors ($P < 0.05$).

RESULTS AND DISCUSSION

Body weights of MT-III null mice and wild-type mice were monitored upon exposure to Cd for 67 weeks. MT-I/II null mice were also exposed to Cd as a reference group. Several studies, including our findings, have suggested that MT-I/II null mice exhibit severe hepatotoxicity compared with wild-type mice upon single administration of Cd (Masters *et al.*, 1994; Liu *et al.*, 1996; Honda *et al.*, 2010a). As expected, MT-I/II null mice died upon 18-week exposure to Cd (Fig. 1B). The body weight of Cd-exposed wild-type mice was significantly lower than that of non-exposed wild-type mice (Fig. 1A). However, Cd-exposed MT-III null mice showed similar body weights as those of non-exposed MT-III null mice (Fig. 1C).

To determine hepatotoxicity, the activities of GOT and GPT in serum were examined and histopathologic analyses on the liver conducted. Activities of GOT and GPT in the serum of wild-type mice were slightly increased upon Cd exposure, but severe histologic changes in the liver upon Cd exposure were not observed (Figs. 2, 3). Conversely, long-term exposure to Cd did not affect the activities of GOT or GPT in the serum or histologic changes in the liver of MT-III null mice (Figs. 2, 3). MT-III null mice seemed to eliminate the hepatotoxicity elicited by long-term exposure to Cd compared with wild-type mice. The effect of MT-III on the hepatotoxicity wrought by long-term exposure to Cd may not be extensive because long-term exposure to Cd resulted in mild hepatotoxicity in wild-type mice.

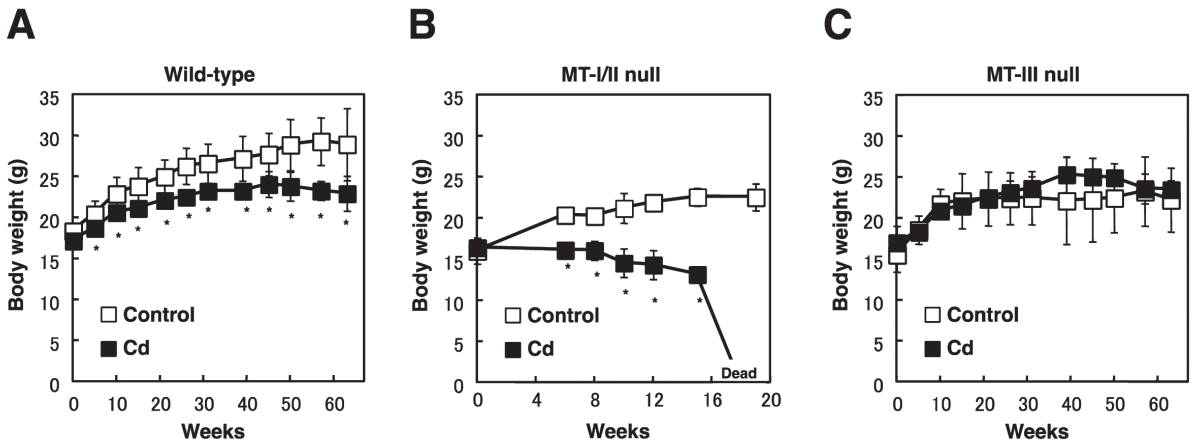

Fig. 1. Changes in body weight in mice upon chronic exposure to Cd. Wild-type mice (A) and MT-III null mice (C) were bred with a diet containing 300 ppm Cd for 67 weeks. (B) MT-I/II null mice were bred with a diet containing 300 ppm Cd for 18 weeks. Values are the mean ± S.D. (n = 3-5). *Significantly different from the corresponding control group, $P < 0.05$.

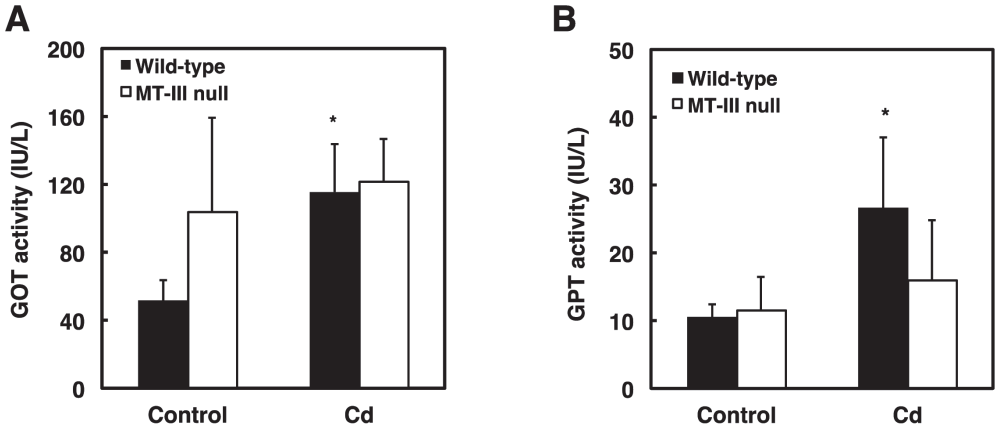

Fig. 2. Activities of GOT and GPT in the serum of wild-type mice and MT-III null mice upon chronic exposure to Cd. Activities of GOT (A) and GPT (B) were examined after exposure to 300 ppm Cd for 67 weeks. Values are the mean ± S.D. (n = 3-5). *Significantly different from the corresponding control group, $P < 0.05$.

Cd exposure induces expression of MT-I and MT-II in the liver of mice to aid elimination of Cd toxicity (Nordberg *et al.*, 2007). Long-term exposure to Cd induced high mRNA levels of MT-I and MT-II in the liver of wild-type mice and MT-III null mice (Fig. 4A, B). These results suggest that long-term exposure to Cd may contribute similar sensitivity in the liver of MT-III null mice as that of wild-type mice because expression of MT-I and MT-II was induced in the liver of MT-III null mice.

MT-III was discovered as a growth inhibitory factor in the brain (Uchida *et al.*, 1991). Moreover, MT-III expression has been identified in the testis, epididymis, prostate, uterus, ovary, kidney, intestine and tongue (Moffatt and Séguin, 1998; Hozumi, *et al.*, 2008). However, MT-III is hardly expressed in the liver of wild-type mice (Moffatt and Séguin, 1998; Honda *et al.*, 2010a). Our previous study demonstrated that MT-III null mice are resistant to Cd-induced acute hepatotoxicity, though MT-I/II null mice are highly sensitive to Cd-induced acute hepatotoxicity (Honda *et al.*, 2010a). Moreover, it was revealed that although the Cd concentration in the liver of the MT-I/II null mice was significantly lower than that of the wild-type mice, the hepatic Cd concentrations were not different between the MT-III null mice and wild-type mice (Honda *et al*., 2010a). These findings suggest that MT-III may be an accelerative factor, and MT-I and MT-

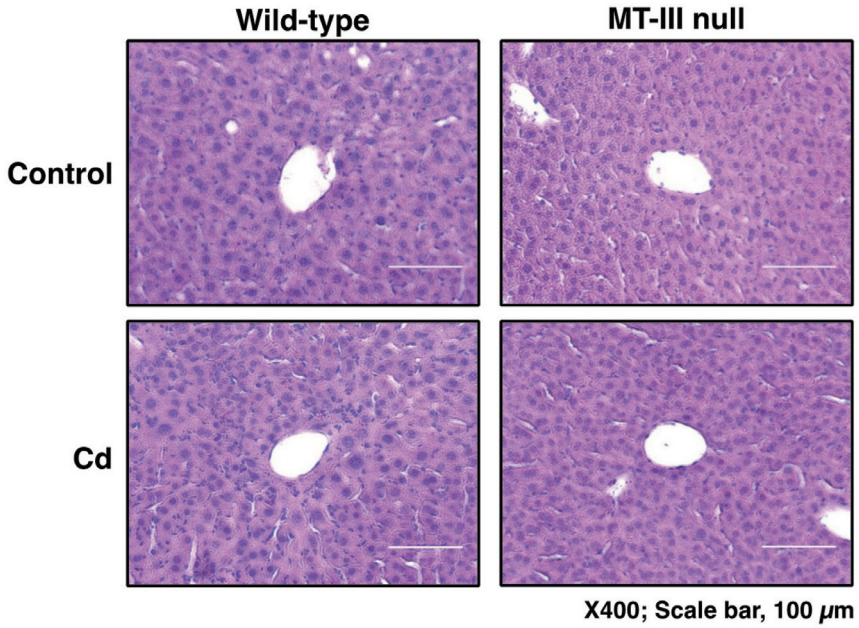

Fig. 3. Histopathologic changes in the liver of wild-type mice and MT-III null mice upon chronic exposure to Cd. Wild-type mice and MT-III null mice were bred with a diet containing 300 ppm Cd for 67 weeks. Histopathologic changes in the liver were examined by staining (hematoxylin and eosin). Original magnification: × 400. Scale bar: 100 μm.

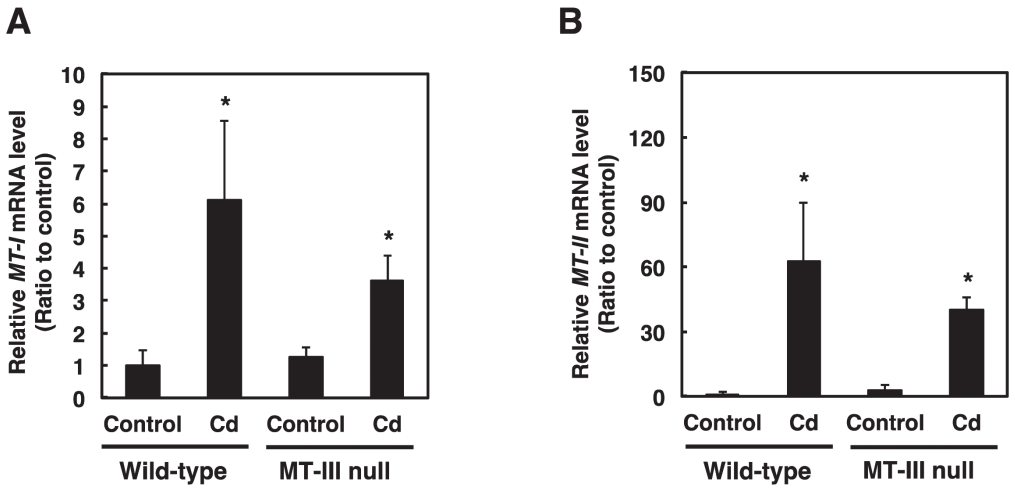

Fig. 4. Effects of chronic exposure to Cd on mRNA levels of MT-I and MT-II in the liver of wild-type mice and MT-III null mice. Wild-type mice and MT-III null mice were bred with a diet containing 300 ppm Cd for 67 weeks. mRNA levels of MT-I (A), MT-II (B) were determined by real-time RT-PCR. mRNA levels were normalized with *β-actin*. Values are the mean ± S.D. (n = 3-5). *Significantly different from the corresponding control group, $P < 0.05$.

II may be defensive factors, in Cd-induced acute hepatotoxicity. However, upon long-term exposure to Cd, sufficient amounts of MT-I and MT-II are induced so that Cd hepatotoxicity is eliminated in wild-type mice and MT-III null mice.

MT-III can prevent oxidative stress by scavenging free radicals (Montoliu *et al*., 2000; You *et al.*, 2002; Uchida *et al.*, 2002). Furthermore, MT-III is thought to protect against nerve damage. MT-III null mice are highly sensitive to seizures induced by kainic acid (Erickson *et al.*,

1997), and to neuronal damage after transient focal cerebral ischemia (Koumura *et al.*, 2009). However, MT-III has a yet-unknown effect on the defense mechanism against hepatotoxicity caused by acute or chronic exposure to Cd. Further studies need to evaluate the effect of MT-III on Cd-induced hepatotoxicity.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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