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Original Article

Resistance of metallothionein-III null mice to cadmium-induced acute hepatotoxicity

Akiko Honda[1,4], Hiroaki Komuro[1], Tatsuya Hasegawa[2], Yoshiyuki Seko[2], Akinori Shimada[3], Hisamitsu Nagase[4], Isao Hozumi[5], Takashi Inuzuka[5], Hideaki Hara[6], Yasuyuki Fujiwara[1] and Masahiko Satoh[1]

[1]Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan
[2]Department of Environmental Biochemistry, Yamanashi Institute of Environmental Sciences, 5597-1 Kenmarubi, Kamiyoshida, Fujiyoshida, Yamanashi 403-0005, Japan
[3]Department of Veterinary Pathology, Faculty of Agriculture, Tottori University, 4-101 Koyama-minami, Tottori City, Tottori 680-0945, Japan
[4]Laboratory of Hygienic Chemistry and Molecular Toxicology, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan
[5]Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan
[6]Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan

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ABSTRACT — We examined the sensitivity of metallothionein (MT)-III null mice to cadmium (Cd)-induced acute hepatotoxicity. MT-I/II null mice were also used to compare Cd toxicities between MT-III null mice and MT-I/II null mice. Male MT-I/II null mice, MT-III null mice and wild-type mice were given s.c. injection of Cd (5-20 μmol/kg) and then the blood and liver were collected from each mouse under ether anesthesia at 2 days after the administration. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities elevated by injection of Cd were significantly higher in the MT-I/II null mice than in the wild-type mice. In the MT-III null mice, ALT and AST activities were not elevated following the injection of Cd. Further, marked morphological changes such as necrosis of hepatocytes, severe hemorrhage and congestion were observed by injection of Cd in both MT-I/II null mice and wild-type mice, whereas the degree of injury was found to be more extensive in MT-I/II null mice. In contrast, only occasional damage was observed in the liver of MT-III null mice treated with the same dose of Cd. These morphological observations were consistent with the results of ALT and AST activities. In the present study, it was clearly found that MT-III null mice were resistant to Cd hepatotoxicity, although MT-I/II null mice were sensitive to its toxicity. MT-III may be an accelerative factor in Cd-induced acute hepatotoxicity.

Key words: MT-III, MT-I/II, Cd, Hepatotoxicity, Knockout mice

INTRODUCTION

Cadmium (Cd) is an environmental contaminant that can adversely affect a number of tissues, including the liver, kidney, lung, testis and bone. In particular, the major target organ of acute and chronic Cd toxicity is the liver; Cd causes parenchymal cell swelling, degeneration and necrosis in the liver (Nordberg *et al.*, 2007, 2009; Rikans and Yamano, 2000).

Metallothionein (MT) is a cysteine-rich low-molecular-weight protein with a high affinity for metals such as Cd and mercury and is induced by a number of stimuli including various metals (Klaassen *et al.*, 1999). Four major isoforms of MT have been identified, MT-I and MT-II are expressed ubiquitously in the tissues, MT-III expression mainly occurs in the brain (Palmiter *et al.*,

Correspondence: Masahiko Satoh (E-mail: masahiko@dpc.agu.ac.jp)

1992), and MT-IV is located in the stratified squamous epithelia (Quaife *et al*., 1994). MT-I and MT-II have been demonstrated to play an important role in protection from Cd toxicity. MT-I/II null mice with disrupted genes of MT-I and MT-II have exhibited high sensitivity to Cd toxicity, such as hepatotoxicity, nephrotoxicity, bone toxicity, hematotoxicity and immunotoxicity (Liu *et al.*, 1996, 1998, 1999 and 2000; Habeebu *et al.*, 2000a, 2000b). Concerning Cd-induced lethal toxicity, Park *et al*. (2001) have reported that the LD_{50} of Cd in wild-type mice was 7-fold higher than that in MT-I/II null mice. In addition, several studies have demonstrated that MT-I/II null cells are also sensitive to Cd cytotoxicity (Lazo *et al.*, 1995; Zheng *et al*., 1996; Kondo *et al.*, 1999).

MT-III was originally discovered as a growth inhibitory factor that was deficient in the brain of patients with Alzheimer's disease (Uchida *et al*., 1991). Recently, the expression of MT-III has also been identified in the testis, epididymis, prostate, uterus, ovary, kidney, intestine and tongue (Moffat and Séguin, 1998; Hozumi *et al*., 2008). Despite the fact that MT-III is found in various tissues, most studies have focused on understanding the role of MT-III in the brain. A few reports have shown that MT-III expression in human proximal tubule cells is induced by Cd treatment (Garrett *et al.*, 2002), and MT-III could affect Cd-induced apoptosis and necrosis in human proximal tubule cells (Somji *et al*., 2004). Thus, MT-III is possibly associated with Cd toxicity. However, no studies have examined the role of MT-III in Cd toxicity *in vivo*. In the present study, we examined the susceptibility of MT-III null mice to Cd-induced acute hepatotoxicity. In addition, MT-I/II null mice were also used to compare Cd toxicities between MT-III null mice and MT-I/II null mice.

MATERIALS AND METHODS

Chemicals

Cadmium chloride, 10% neutral buffered formalin solution, and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals

MT-I/II null mice (Masters *et al*., 1994) and MT-III null mice (Erickson *et al.*, 1997) possessed null mutation of MT-I/II and MT-III, respectively. MT-I/II null mice, MT-III null mice and wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and routinely bred in the laboratory animal facility of Aichi Gakuin University. MT-I/II null mice and MT-III null mice were of the 129/Sv strain. Age- and sex-matched 129/Sv mice were used as wild-type controls. All strains of mice were housed in cages in ventilated animal rooms at a controlled temperature of 23 ± 1°C with a relative humidity. They were maintained on standard laboratory chow and tap water *ad libitum*, and received humane care throughout the experiment according to the guidelines established by the Aichi Gakuin University.

Treatments

Ten-twelve weeks old male MT-I/II null mice, MT-III null mice and wild-type mice were randomly assigned to control and experimental groups, with 4-6 mice per group. Each experimental group was given a single subcutaneous injection of Cd at a dose of 5, 10 or 20 μmol/kg and then mice were sacrificed under diethyl ether anesthesia at 2 days after the administration. The blood and liver were collected from each mouse to evaluate the hepatotoxicity, Cd concentration and elution profiles in the liver.

MT-I/II null mice, MT-III null mice and wild-type mice were subcutaneously injected with Cd at a dose of 5 μmol/kg in order to characterize the distribution of Cd in the liver. The liver was removed from the mice under diethyl ether anesthesia at 1, 3 and 7 days after the injection of Cd.

MT-III null mice and wild-type mice were subcutaneously administered with Cd at a dose of 20 μmol/kg. The liver was removed from the mice under diethyl ether anesthesia at 6 and 24 hr after the injection of Cd for evaluation of MT-I, MT-II and MT-III mRNA expression.

Analyses

AST and ALT activities

To evaluate the hepatotoxicity, the automatic dry-chemistry analyzer system (Spotchem EZ SP-4430; Arkray, Kyoto, Japan) was used to determine the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum.

Histopathology

The liver was fixed in 10% neutral buffered formalin solution and embedded in paraffin. Deparaffinized tissue sections of 5 μm thickness were stained with hematoxylin and eosin.

Cd concentration

Prior to the analysis of Cd concentrations, the liver was digested with nitric acid and hydrogen oxidate. After digestion, the inorganic residues were dissolved in ultrapure water, and metal analysis was carried out using inductively coupled plasma-mass spectrometry (ICP-MS,

ICP model; Agilent Technologies 4500; Santa Clara, CA, USA).

HPLC analysis

The liver was homogenized in 4 volumes of saline, and the homogenates were ultracentrifuged at 4°C for 60 min at 105,000 × *g*. The resultant supernatant was filtered and applied to 100 µl of hepatic cytosol through TSK-Gel G3000SW column (TOSOH, Tokyo, Japan), and eluted with 50 mM bicine ammonia (pH 8.2) at a flow rate of 0.8 ml/min (Agilent Technologies 4500). Cd in the eluted sample was detected by ICP-MS connected to the high-performance liquid chromatography (HPLC) column.

MT-I, MT-II and MT-III mRNA level

The liver was obtained from MT-III null and wild-type mice at 6 and 24 hr after the injection of Cd (20 µmol/kg). Total RNA was isolated from the liver using QuickGene RNA tissue kit S II (Fujifilm, Tokyo, Japan). RT-PCR was performed to determine the gene expression of MT-I, MT-II, MT-III and β-actin. Briefly, RT-PCR was performed using a reaction mixture containing the primers listed in Table 1. Reactions were performed with 5 sec denaturation at 95°C and 30 sec elongation with annealing at 60°C, for a total of 27 cycle. The PCR products were electrophoresed on a 3% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

The data are presented as means ± standard deviation (S.D.) for each experimental group (n = 4-6). Statistical analyses were performed using Student's *t*-test or one-way analyses of variance (ANOVA) followed by Bonferroni's test for *post-hoc* comparison. Differences between the groups were considered significant at $P < 0.05$.

RESULTS

The ALT and AST activities, indicators of hepatotoxicity, in the serum of MT-I/II null mice, MT-III null mice and wild-type mice injected with Cd are shown in Fig. 1. ALT and AST activities were elevated in the serum of both MT-I/II null mice and wild-type mice injected with Cd (20 µmol/kg) ; moreover, these elevations were significantly greater in the MT-I/II null mice than in the wild-type mice. In the MT-III null mice, ALT and AST activities were not elevated following the injection of Cd.

The resistance to hepatotoxicity of Cd in the MT-III null mice was further confirmed by histopathological observations (Fig. 2). In the liver of MT-I/II null mice and wild-type mice, marked morphological changes, such as the necrosis of hepatocytes with neutrophil infiltration, severe hemorrhage and congestion were observed following the injection of Cd at a dose of 20 µmol/kg. Moreover, the liver injury in the MT-I/II null mice injected with Cd was more severe as compared with that in the wild-type mice. In the MT-III null mice, occasional foci of hepatocytic necrosis with a few neutrophils and signs of focal congestion were observed following the injection of Cd at the same dose.

Cd concentrations in the liver of MT-I/II null mice, MT-III null mice and wild-type mice after Cd injection are shown in Fig. 3. Cd concentrations in the liver of MT-III null mice and wild-type mice were increased by Cd injection in a dose-dependent manner. However, the hepatic Cd concentrations in the Cd-injected mice did not differ significantly between the MT-III null mice and wild-type mice. On the other hand, Cd concentration in the liver of the MT-I/II null mice was lower than that in the wild-type mice at all doses of Cd. Fig. 4 shows the time course of Cd concentrations in the liver of MT-I/II null mice, MT-III null mice and wild-type mice after the injection of Cd at a dose of 5 µmol/kg which did not cause hepatotoxicity. At 1, 3 and 7 days after the injection, Cd level in the liver of MT-I/II null mice was lower than that in wild-type mice, whereas Cd level in the liver of MT-III null mice was similar to that in wild-type mice.

The elution profile of the liver cytosols from MT-I/II null mice, MT-III null mice and wild-type mice is shown in Fig. 5. In the MT-III null mice and wild-type mice, Cd-MT-I/MT-II complex was found, and both the elution profiles were similar. However, almost all the Cd in the hepatic cytosol of MT-I/II null mice bound high-molecu-

Table 1. PCR primer sequences for metallothionein isoforms and β-actin

Name	Primer (5′-3′)	Product Size (bp)
MT-I	Forward: GGTCCTCTAAGCGTCACCAC	102
	Reverse: GAGCAGTTGGGGTCCATTC	
MT-II	Forward: CCTGTGCCTCCGATGGAT	153
	Reverse: ACTTGTCGGAAGCCTCTTTG	
MT-III	Forward: CTGAGACCTGCCCCTGTC	181
	Reverse: TTCTCGGCCTCTGCCTTG	
β-actin	Forward: GATCTGGCACCACACCTTCT	138
	Reverse: GGGGTGTTGAAGGTCTCAAA	

lar weight protein.

As shown in Fig. 6, the MT-I, MT-II and MT-III mRNA levels were determined in the liver of MT-III null mice and wild-type mice after a dose of 20 μmol/kg. The MT-I and MT-II mRNA levels in the liver of both the strains of mice were increased at 24 hr and 6 hr later after Cd injection, respectively. There were no differences in the MT-I and MT-II mRNA levels between the MT-III null mice and wild-type mice after Cd injection. The MT-III mRNA levels in the liver of both strains of mice were below the detection limit and could not be induced by Cd injection (Fig. 6).

DISCUSSION

Various studies have reported that MT-I/II null mice have an increased sensitivity to the acute and chronic toxicities caused by Cd (Klaassen *et al.*, 2009). The present study also demonstrated that MT-I/II null mice were highly sensitive to Cd-induced acute hepatotoxicity. These results suggest that MT-I/II acts as a biological defense factor against Cd toxicity. Further, our present study demonstrated for the first time that MT-III null mice were resistant to Cd-induced acute hepatotoxicity (Figs. 1 and 2). In a recent *in vitro* study, Somji *et al.* (2004) have demonstrated that HK-2 cells transfected with MT-III were more sensitive to Cd cytotoxicity. These *in vitro* and *in vivo* studies clearly indicate, therefore, that MT-III enhances Cd toxicity.

MT-III can act as free radical scavenger and prevent oxidative stress (Montoliu *et al.*, 2000; You *et al.*, 2002; Uchida *et al.*, 2002). MT-III null mice have been used to directly evaluate the biological functions of MT-III in nervous system damage. Erickson *et al.* (1997) have indicated that MT-III null mice were highly susceptible to seizures induced by kainic acid. Koumura *et al.* (2009) have also demonstrated that MT-III null mice showed an increased sensitivity to neuronal damage after transient focal cerebral ischemia. In contrast, previous studies using MT-III null mice have shown that MT-III inhibits peripheral nerve regeneration and is not involved in protecting neurons from damage caused by a focal cryolesion (Carrasco *et al.*, 2003; Ceballos *et al.*, 2003). Further studies involving MT-III null mice are essential to clarify the role of MT-III in nervous system damage.

In the present study, it was interestingly found that the sensitivity to Cd-induced acute hepatotoxicity differed between MT-III null mice and wild-type mice although MT-III is not expressed in the liver of wild-type mice. The mechanisms involved in the acceleration of Cd hepatotoxicity by MT-III remain unclear. The present study indicated that the differences in the sensitivity to Cd hepatotoxicity between MT-III null mice and wild-type mice is not due to the greater induction of MT-I and MT-II by Cd, the accumulation of Cd, or the binding of Cd to MT-I and MT-II in the liver of MT-III null mice injected with Cd. Moreover, it is considered that the endogenous MT-III level, which is not detectable in the liver, may be

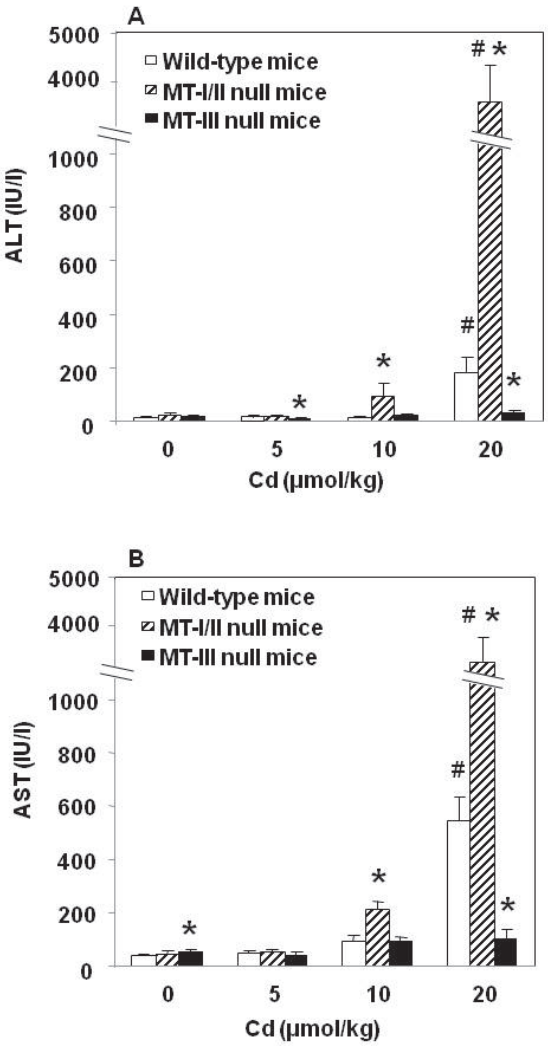

Fig. 1. ALT and AST activities in the serum of MT-I/II null mice, MT-III null mice and wild-type mice injected with Cd. ALT and AST activities were determined at 2 days after Cd injection (5, 10 or 20 μmol/kg). A: ALT activity, B: AST activity. Values are mean ± S.D. for 4-6 mice.

[#] Significantly different from the corresponding control group ($P < 0.05$).

[*] Significantly different from the corresponding wild-type mice ($P < 0.05$).

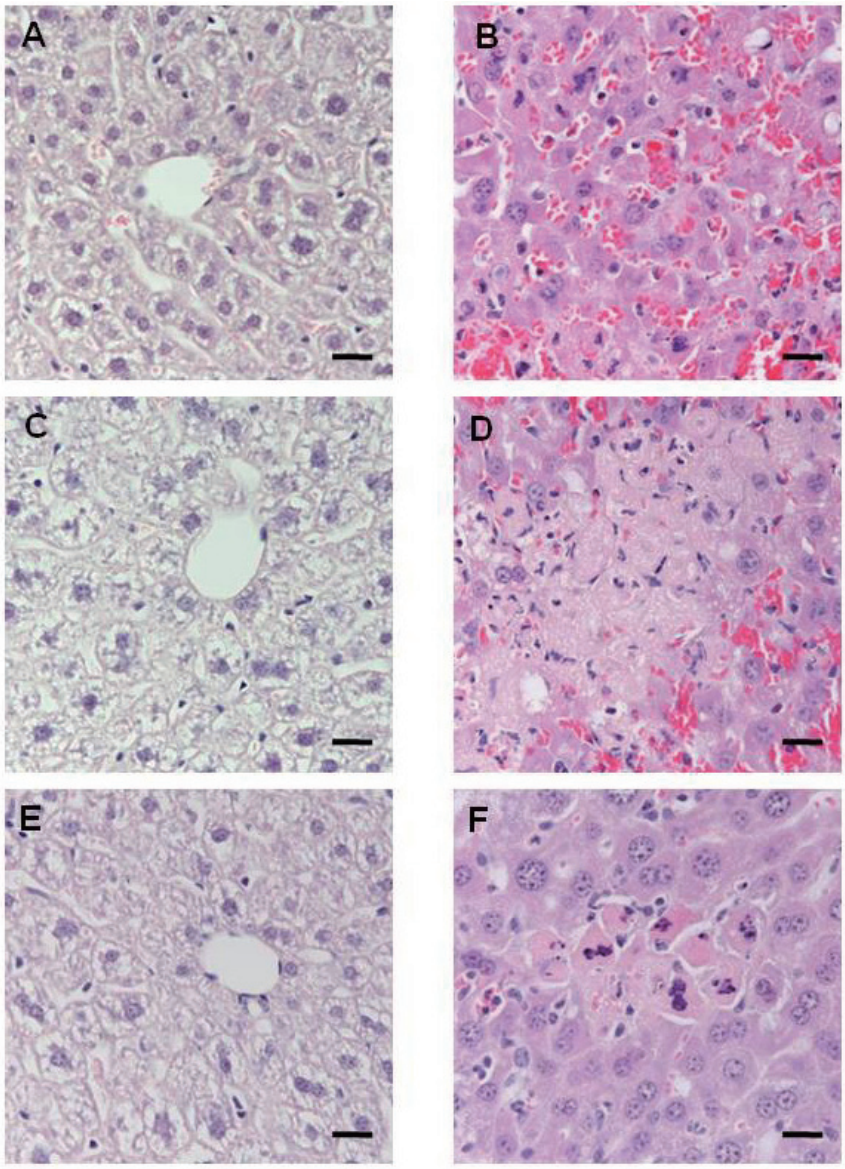

Fig. 2. Histopathological changes in the liver of MT-I/II null mice, MT-III null mice and wild-type mice injected with Cd. The liver was stained with hematoxylin and eosin. Histopathological changes were evaluated at 2 days after Cd injection (20 μmol/kg). A: Saline-injected control wild-type mice, B: Cd-injected wild-type mice, C: Saline-injected control MT-I/II null mice, D: Cd-injected MT-I/II null mice, E: Saline-injected control MT-III null mice, and F: Cd-injected MT-III null mice. Bar = 10 μm.

increased by Cd exposure; consequently, Cd-induced MT-III may accelerate the liver damage caused by Cd. However, our results showed that MT-III expression in the liver of wild-type mice was not detected after Cd injection (Fig. 6).

In conclusion, we found that Cd-induced acute hepatotoxicity was enhanced by the presence of MT-III but greatly inhibited by that of MT-I and MT-II. Additionally, this effect of MT-III did not appear to be related to Cd accumulation or the binding of Cd to MT-I/MT-II in the liver. Our study thus suggests that MT-III may be one of the factors responsible for Cd injury including acute hepatotoxicity.

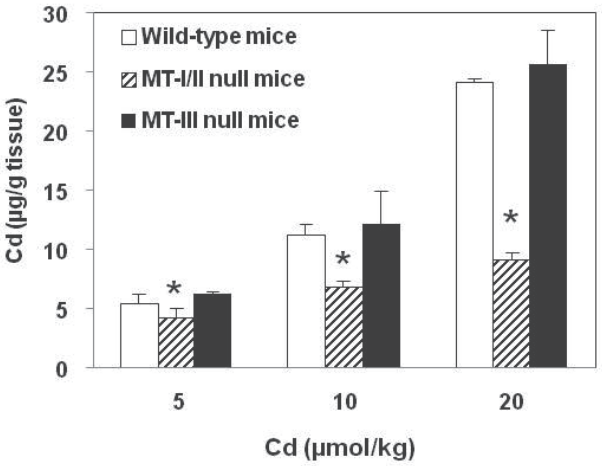

Fig. 3. Cd concentrations in the liver of MT-I/II null mice, MT-III null mice and wild-type mice injected with Cd. Cd concentrations were determined at 2 days after Cd injection (5, 10 or 20 μmol/kg). Values are mean ± S.D. for 4-6 mice.
* Significantly different from the corresponding wild-type mice ($P < 0.05$).

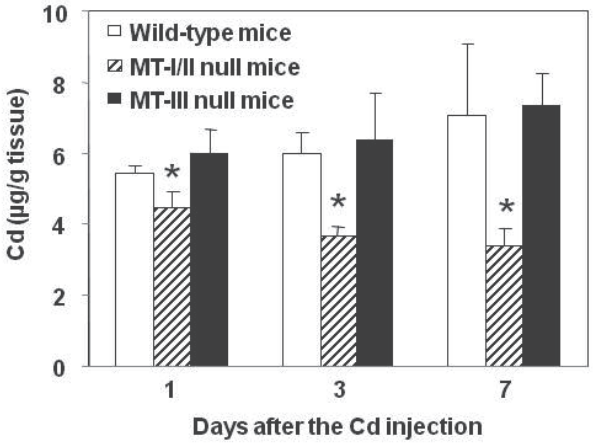

Fig. 4. Cd concentrations in the liver of MT-I/II null mice, MT-III null mice and wild-type mice at various times after Cd injection. Cd concentrations were determined at 1, 3 and 7 days after Cd injection (5 μmol/kg). Values are mean ± S.D. for 4-6 mice.
* Significantly different from the corresponding wild-type mice ($P < 0.05$).

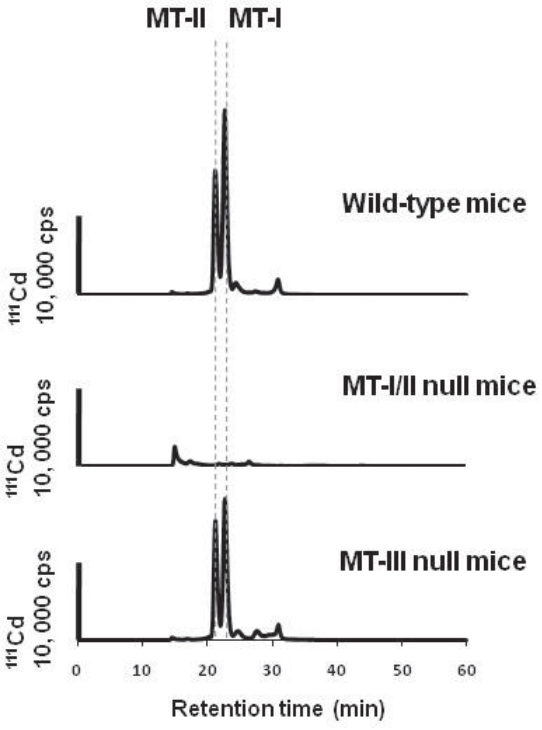

Fig. 5. The elution profiles of Cd in the hepatic cytosol of MT-I/II null mice, MT-III null mice and wild-type mice injected with Cd. The elution profiles of Cd were determined at 2 days after Cd injection (20 μmol/kg) using HPLC-ICP/MS analysis.

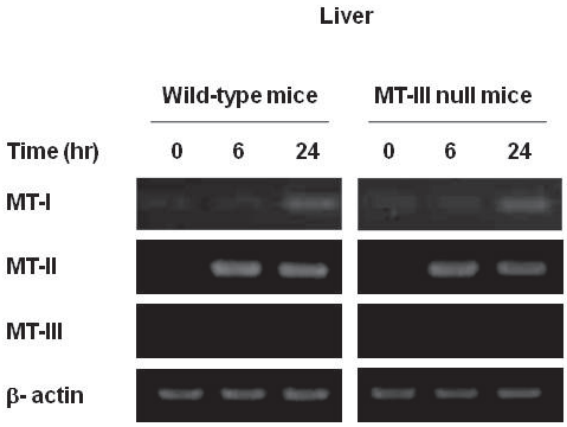

Fig. 6. MT-I, MT-II and MT-III mRNA levels in the liver of MT-III null mice and wild-type mice injected with Cd. The MT mRNA levels were determined at 6 and 24 hr after Cd injection (20 μmol/kg).

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