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Subcellular changes of essential metal shown by in-air micro-PIXE in oral cadmium-exposed mice

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Abstract To clarify the relation of essential metals to cadmium (Cd) toxicity, we evaluated metallothionein expression and analyzed the subcellular distribution of essential metals using in-air micro-Particle-Induced X-ray Emission (PIXE). Four mice were dosed orally with 100 mg/L of Cd in drinking water for 1.5 or 2 years. Frozen samples of organs were used for micro-PIXE analysis and formalin-fixed samples were used for metallothionein staining. Immunohistochemically, metallothionein induction by 1.5y-Cd exposure was higher in the renal cortex than in the liver. Metallothionein expression was reduced after 2y-Cd administration compared to the 1.5y-Cd-exposed mice. Cd-induced tissue damage became marked in the 2y-Cd-exposed mice compared to the 1.5y-Cd-exposed mice, in which nephrotoxicity was more prominent than hepatotoxicity. Cd yield was higher in the renal cortex of the 2y-Cd-exposed mouse than in that of the 1.5y-Cd-exposed mouse, whereas no such increasing tendency was found in the liver. Compared to the control, the Cd-exposed mice markedly accumulated zinc in the liver and renal cortex. In the Cd-exposed mice, iron was mildly accumulated in the renal cortex and was slightly deprived in the liver. Elemental maps showed that a large amount of Cd was spatially combined with zinc in the 1.5y-Cd mouse. Free Cd became abundant in the 2y-Cd-exposed mouse. In addition, a small amount of Cd was colocalized with iron. The data suggest that zinc may contribute to protect against oral-administrated Cd toxicity, and impaired induction of MT may participate in hepato-nephrotoxicity of the 2y-Cd-exposed mouse.

Keywords Cadmium · In-air micro-PIXE · Zinc · Iron · Metallothionein

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Introduction

Cadmium (Cd), an industrial and environmental pollutant, is toxic and carcinogenic to a wide range of organs (Patrick 2003; Waalkes 2003;

Valko et al. 2005). Cd toxicity is very dependent on the dose, route, and duration of exposure. Anderson O et al (1988) reported that the fractional absorption increased with increasing parenteral doses of Cd. The relative Cd deposition in brain, testis and intestines decreased with increasing dose, whereas the relative liver deposition increased with dose. After oral Cd is absorbed through the digestive tract, Cd can induce and bind to low molecular weight (6–7 kDa) protein metallothionein (MT) in the liver (Kagi and Kojima 1987; Cherian and Cahn 1993; David and Cousins 2000). MT/Cd complex is slowly released over time from the liver and circulates to the kidneys where it can accumulate in renal tissue. The main pathologic changes related to chronic Cd toxicity of renal disease are reflective of Cd concentration in the kidney and the alteration of renal function (Nordberg et al. 1994; Mitsumori et al. 1998; Jarup et al. 1998; Wlostowski et al. 2000; Brzoska et al. 2003).

The mechanisms of Cd toxicity are not completely understood, but some of the specific changes that lead to tissue damage and death in chronic exposure have been related to oxidative stress and thiol depression (Ercal et al. 2001; Martelli and Moulis 2004; Hansen et al. 2006). Cd is assumed to produce histopathological changes indirectly, through iron (Fe)-dependent oxidative processes (Casalino et al. 1997; Watjen et al. 2004; Martelli et al. 2006). In addition, Cd may cause changes in the homeostasis of zinc (Zn) and copper (Cu) because of their similar ionic radius (Oishi et al. 2000; Pourahmad et al. 2003; Noel et al. 2004; Martelli and Moulis 2004; Valko et al. 2005; Hansen et al. 2006). Changes in Zn and Cu homeostasis have been found to be closely associated with MT levels, which serve as radical scavengers and detoxify toxic metals (Klassen et al. 1999). Thus, the intervention of Cd with tissue essential metals may play a critical role in Cd toxicity; however, the subcellular distribution of essential metals in Cd toxicity is poorly understood.

An in-air micro-Particle Induced X-ray Emission (PIXE) system was developed in a TIARA facility, JAEA Takasaki (Sakai et al. 2002). The in-air micro-PIXE analyzing technique is the only apparatus measuring and visualizing the spatial distribution and dynamics of various elements in a single cell in the atmosphere within a spatial resolution of 1 μm. Using this apparatus, we detected Cd distribution in the liver of an experimental animal (Sakai et al. 2005). The net count ratio of metal to Sulfur (S) calculated by micro-PIXE well correlated with tissue metal concentration so that in-air micro-PIXE is reliable for evaluating the trace element level (Nagamine et al. 2006).

In the present study, we analyzed the intracellular changes of metal elements in chronic Cd-exposed mice using in-air micro-PIXE. In addition, we estimated the relation of MT induction to Cd-induced liver and kidney injury.

Materials and methods

Animal and Cd treatment

Six ICR female mice (4-weeks-old) were kept in the animal facility of Gunma University, with 12 h light/dark regime and at constant temperature (25°C). Mice were allowed *ad libitum* access to standard MF diet purchased from Oriental Yeast Co., Ltd.

Four mice were allowed free access to water with Cd added at a concentration of 100 mg/L as $CdCl_2$; two mice were exposed to Cd for 1.5y (1.5y-Cd mouse) and two mice for 2.0y (2y-Cd mouse). The other two mice were allowed free access to tap water without Cd for 1.5y (control). The amount of water consumed by each mouse was carefully recorded every week throughout the entire experiment; the lifetime Cd uptake was up to 190 mg in the 1.5y-Cd mouse and 230 mg in the 2y-Cd mouse. At the end of exposure, mice were sacrificed, and the liver and kidney were removed immediately. Tissues were divided into two specimens, one was stored frozen at –80°C for micro-PIXE analysis and the other was fixed in 10% formalin solution for 4 h and embedded in paraffin.

All mice were treated under the guidelines for the care and use of laboratory animals of Gunma University.

Histopathological examination

Paraffin-embedded specimens were cut by a microtome into 3 μm-thick sections, and then stained with hematoxylin and eosin. Histological changes were assessed by light microscopy.

Metallothionein staining

MT protein in paraffin-embedded specimens was stained immunohistochemically by a modified method as previously described (Nagamine et al. 2006). In brief, liver and kidney specimens were incubated in 0.3% hydrogen peroxide/methanol solution to block the intrinsic peroxidase reaction. The specimens were heated in 0.01 M citrate buffer for 5 min in a microwave oven. They were subsequently incubated with rabbit polyclonal anti-MT antibody for 2 h. Histofine Simple Stain MAX-PO (Nichirei Co.) was used as the secondary antibody, and reacted for 1 h. To develop color, 0.02% 3,3′-diamino-benzidine (Dojindo Laboratories) was used. The MT polyclonal rabbit antibody used in this study recognizes both MT-I and MT-II isoforms of human, rat, mouse, or rabbit MT.

In-air micro-PIXE analysis in tissue samples

In addition to a control, two mice exhibiting representative histological changes following 1.5y and 2y-Cd administration were used for micro-PIXE analysis.

The frozen liver and kidney (renal cortex) specimens were cut by a cryostat into 15 μm frozen sections. Sections were placed onto 20 μm-thick Mylar film and immediately fixed to a sample holder.

A 3.0 MeV proton beam, 1 μm beam spot size, accelerated by the TIARA single-ended accelerator at JAEA-Takasaki, was used to analyze subcellular elemental distribution in the tissue samples. The periods and dimensions of the scan were set at 60 min and 70×70 μm^2 areas, respectively. The precise conditions of measurement were reported previously (Sakai et al. 2005). Net count of the element yield was calculated by PC Software program. As the sulfur (S) count is regarded as representative of the whole cell number (Bara et al. 1996), the net count ratios of Cd, Zn, Fe and Cu to S were calculated. In-air micro-PIXE analysis was performed three times for each sample.

Statistical analysis

The net count ratios of Cd, Zn, Fe and Cu to S were expressed as the means ±SD. For comparison for more than two groups, data were analyzed by a one-way ANOVA, followed by Turky test for multiple comparisons. Probabilities of $P < 0.05$ were regarded as statistically significance.

Results

Histological findings of the renal cortex and liver (Fig. 1)

Chronic Cd exposure caused renal injury with the degeneration of tubular epithelia, dilation of tubular lumen and their regenerative proliferation exhibited an irregular arrangement. Renal pathology was mild in the 1.5y-Cd mice and became prominent in the 2y-Cd mice. Nuclear size disparity was the only pathological change in the liver of the 1.5y-Cd mice. Focal necrosis and lymphocyte infiltration were mild to moderate in the liver of the 2y-Cd mice. Nephrotoxicity was more remarkable than hepatotoxicity in the 2y-Cd mice.

Metallothionein immunostaining (Fig. 2)

MT expression was observed exclusively in the cytoplasm of hepatocytes and renal tubular epithelia, and mildly in the nuclei of cells. MT expression was high in the order of the 1.5y-Cd mouse, 2y-Cd mouse and the control. MT staining was commonly higher in the renal cortex than the liver.

X-ray spectra of tissue samples

The X-ray spectra by in-air micro-PIXE are shown in Fig. 3 (renal cortex) and Fig. 4 (liver). Characteristic X-rays of major elements such as

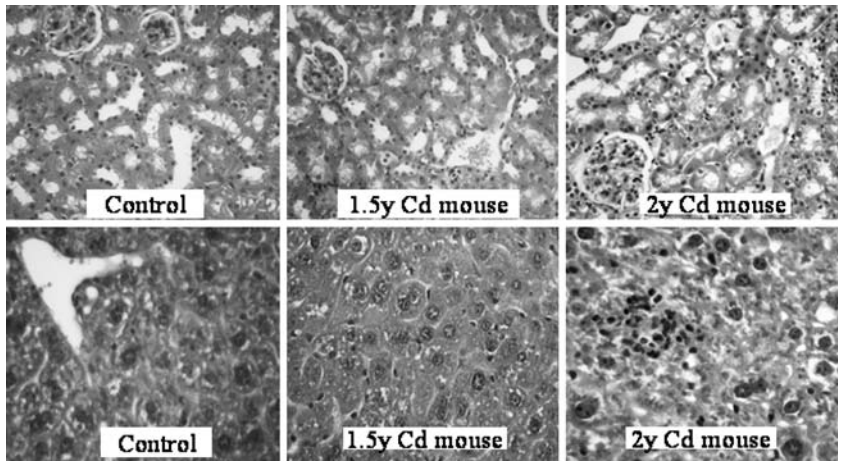

Fig. 1 Histological findings of the renal cortex and liver. Cd toxicity was more prominent in the renal cortex than in the liver. Pathological changes such as the degeneration of tubular epithelia, dilation of tubular lumen and their regenerative proliferation became more obvious in the renal cortex of the 2y-Cd mice than in that of the 1.5y-Cd mice. Nuclear size disparity was shown in the liver of the 1.5y-Cd mice, and mild to moderate focal necrosis and lymphocyte infiltration were found in the liver of the 2y-Cd mice. Upper panel: The renal cortex specimen (HE stain, 200×). Lower panel: The liver specimen (HE stain, 400×)

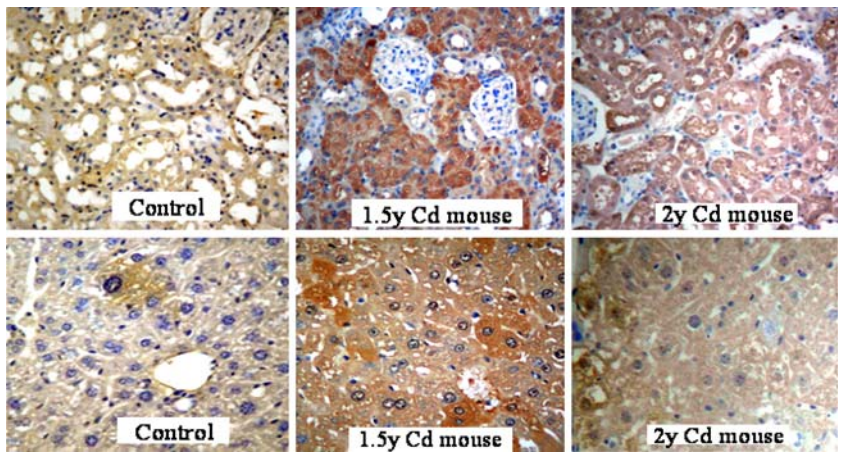

Fig. 2 Metallothionein immunostaining. MT expression was increased in Cd-dosed mice compared to the control, and was commonly higher in the kidney than the liver. In the renal cortex and liver, MT staining was higher in the 1.5y-Cd mouse than the 2y-Cd mouse. Upper panel: The renal cortex specimen (MT staining, 200×). Lower panel: The liver specimen (MT staining, 400×)

phosphorus (P), S, potassium (K), chlorine (Cl), Fe, Zn and Cu were determined by HP-GeX-ray detector (Ortec IGLET, 100 mm^2) in both Cd-exposed and control tissues. The X-ray yield of Cd was provided by Backward X-ray detector (Aptec PS305-D7.5C remodeled) in the renal cortex and liver of Cd-exposed mice (Sakai et al. 2005).

In the renal cortex, Cd yield was higher in the 2y-Cd mouse than the 1.5y-Cd mouse. In the liver, Cd yield did not increase in the 2y-Cd mouse compared to the 1.5y-Cd mouse. As shown in Table 1, the net Cd count ratio in the renal cortex was significantly higher in the 2y-Cd mouse than the 1.5y-Cd mouse. The net Cd count ratio in the liver was similar between the 2y-Cd mouse and

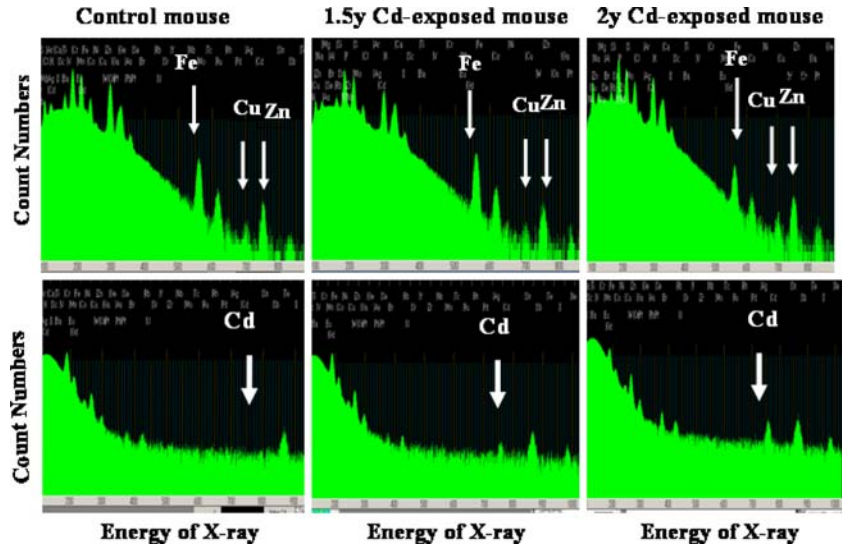

Fig. 3 Representative X-ray spectra by in-air micro-PIXE in the renal cortex. In the renal cortex, Cd yield was higher in the 2y-Cd mouse than the 1.5y-Cd mouse. Cd yield was not detected in the control sample. Zn yield was greater in Cd-exposed mice than the control. Compared to the control levels, Fe yield was slightly high in the 1.5y-Cd mouse. Cu yield was very small in both Cd-exposed and control mice. Upper panel: X-ray spectra using HP-GeX-ray detector. Lower panel: X-ray spectra using Backward X-ray detector

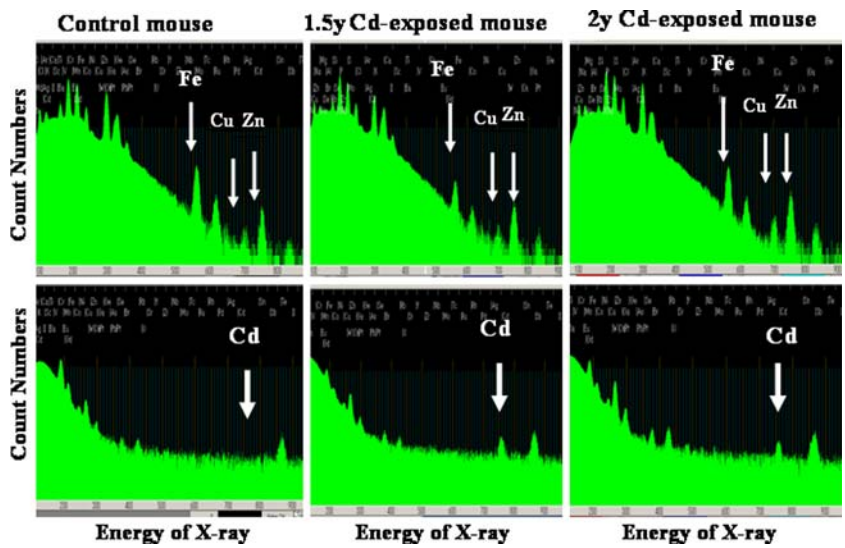

Fig. 4 Representative X-ray spectra by in-air micro-PIXE in the liver. In the liver, Cd yield did not increase in the 2y-Cd mouse compared to the 1.5y-Cd mouse. Cd yield was not detected in the control sample. Zn yield was increased in Cd-exposed mice compared to the control, whereas Fe yield showed an opposite tendency. Cu yield was very small in both Cd-exposed mice and the control. Upper panel: X-ray spectra using HP-GeX-ray detector. Lower panel: X-ray spectra using Backward X-ray detector

the 1.5y-Cd mouse. Cd yield was not detected in the control samples.

Zn yield in the renal cortex was greater in the Cd-exposed mice than in the control, and the net Zn count ratio was also significantly higher ($P < 0.01$) in the former than the latter (Table 1). In the liver, the net Zn count ratio was high in the order of the 2y-Cd mouse, 1.5y-Cd mouse and the

Table 1 Net count ratio of metals to S calculated by micro-PIXE

	Kidney				Liver			
	Cd/S	Zn/S	Fe/S	Cu/S	Cd/S	Zn/S	Fe/S	Cu/S
Control	n.d	0.07 ± 0.01	0.50 ± 0.08	0.02 ± 0.01	n.d	0.08 ± 0.01	2.24 ± 1.98	0.03 ± 0.01
1.5y-Cd mouse	0.05 ± 0.04	0.11[a] ± 0.01	1.08 ± 0.64	0.02 ± 0.01	0.08 ± 0.08	0.12 ± 0.04	0.57 ± 0.16	0.03 ± 0.01
2y-Cd mouse	0.19[c] ± 0.08	0.15[a] ± 0.03	0.68 ± 0.24	0.03 ± 0.01	0.06 ± 0.01	0.15[b] ± 0.03	0.41 ± 0.16	0.02 ± 0.01

In the kidney, the mean Zn/S ratio (%) was significantly higher in Cd-exposed mice than the control. In the liver, the mean Zn/S ratio (%) was significantly higher in 2y-Cd mice than the control

[a] significant difference compared to control ($P < 0.01$)

[b] significant difference compared to control ($P < 0.05$)

[c] significant difference compared to the 1.5y-Cd mouse ($P < 0.05$)

n.d: not determined

control. There was a significant difference ($P < 0.05$) between the 2y-Cd mouse and the control.

Compared to the control, the net Fe count ratio was higher in the renal cortex of the 1.5y-Cd mouse and lower in the liver of Cd-exposed mice, although the difference was not significant (Table 1). The net Cu count ratio was very low in both Cd-exposed mice and the control.

Elemental maps of the renal cortex (Fig. 5) and the liver (Fig. 6)

A two-dimensional P map provides a good representation of the physical shape of the cell, so a counter plot of the P map can be drawn (Nagamine et al. 2006).

In the renal cortex of the control mouse, Zn and Fe were diffusely distributed within the tubular epithelia. By oral Cd administration, Zn was moderately increased and Fe was slightly accumulated in the renal cortex. Cd accumulation was revealed in the tubular epithelia and tubular lumen, which was more prominent in the 2y-Cd mouse than the 1.5y-Cd mouse. A large amount of Cd was combined with Zn, and consequently, free Cd was less in the 1.5y-Cd mouse. By contrast, free Cd became greater in the 2y-Cd mouse, although the relative amount of Cd was combined with Zn. A small amount of Cd was co-localized with Fe in the tubular epithelia (Fig. 5).

In the liver of the control mouse, Zn and Fe were diffusely distributed within the parenchyma. A moderate accumulation of Zn and mild lack of

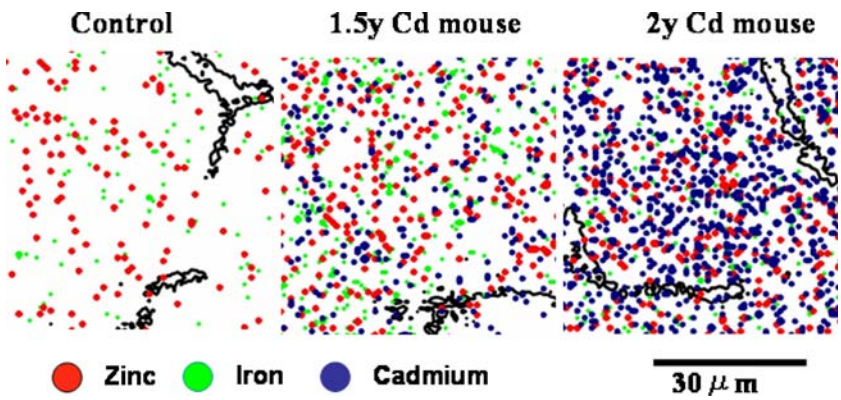

Fig. 5 Elemental maps of the renal cortex. Since the P map provides a good representation of the physical shape of the cell, a counter plat of P map was drawn. In the renal cortex of the control mouse, Fe and Zn were diffusely distributed within the tubular epithelia. In Cd-exposed mice, Cd accumulated in both the tubular epithelia and tubular lumen, and was more prominent in the 2y-Cd mouse than the 1.5y-Cd mouse. Zn was widely distributed in renal tubular epithelia of Cd-exposed mice. Cd was co-localized with a large amount of Zn and small amount of Fe in the 1.5y-Cd mouse. Free Cd became abundant in the 2y-Cd mouse

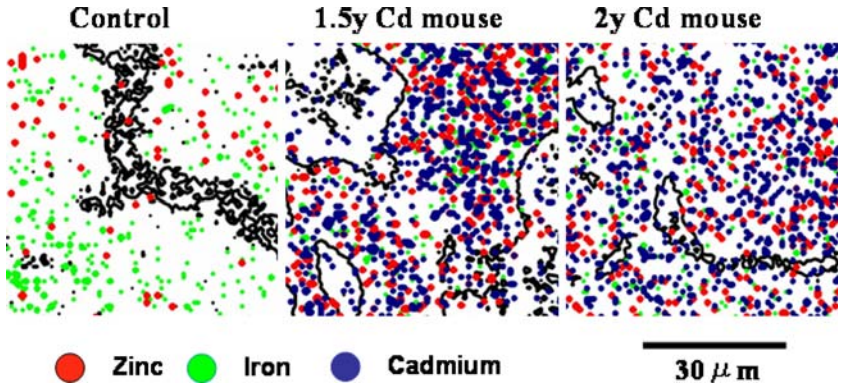

Fig. 6 Elemental maps of the liver. In the liver of the control mouse, Fe and Zn were diffusely distributed within the parenchyma. In Cd-exposed mice, Cd was widely detected in the parenchyma and sinusoids, and Cd accumulation was similar between the 1.5y mouse and 2y-Cd mouse. Zn was markedly accumulated in the cytoplasm in which most Cd spatially combined with Zn in the 1.5y-Cd mouse. Free Cd was increased in the 2y-Cd mouse. Slight deprivation of Fe was observed in Cd-exposed mice

Fe were observed in the liver of Cd-exposed mice. Cd was widely detected in the parenchyma and sinusoids. The degree of Cd accumulation was similar between the 1.5y-Cd mouse and the 2y-Cd mouse. A large amount of Cd was spatially combined with Zn in the 1.5y-Cd mouse and, to a relative extent, in the 2y-Cd mouse. Consequently, free Cd was greater in the 2y-Cd mouse than in the 1.5y-Cd mouse (Fig. 6).

Discussion

Chronic exposure to Cd via food and drinking water is a major human health concern. Chronic Cd exposure interferes with essential metal behavior, mainly Zn, Fe, and Cu (Oishi et al. 2000; Pourahmad et al. 2003; Noel 2004; Martell and Moulis 2004; Valko et al. 2005; Hansen et al. 2006). It is important therefore to determine accurately subcellular changes in metal elements following Cd accumulation so that the risk to health may be identified and minimized. In-air micro-PIXE is a useful technique for visualizing the spatial distribution of various elements in vivo and in vitro (Sakai et al. 2002: Sakai et al. 2005: Nagamine et al. 2006). In the present study, we applied this technology to reveal the intracellular distribution of essential metals in Cd-exposed mice. The net count ratio of Fe, Zn, Cu to S calculated by micro-PIXE were well correlated with tissue metal concentrations determined by ICP-MS, suggesting that in-air micro-PIXE is useful and reliable apparatus for analysis of trace elements (data not shown).

Consistent with prior findings (Nordberg et al. 1994; Mitumori et al. 1998; Wlostowski et al. 2000; Liu et al. 2000; Brzoska et al. 2003), nephrotoxicity was more prominent than hepatotoxicity in mice exposed orally to 100 mg/L of Cd. Most of the total body burden of Cd in animals and humans is associated with MT; thereby, detoxification of MT to Cd is mediated in the liver and probably in the kidney (Klassen et al. 1999; Liu et al. 2000; Martelli et al. 2006). In this study, MT expression was greater in the 1.5y-Cd mouse than in the 2y-Cd mouse, and tissue damages were less in the former than in the later. Compared to the 1.5y-Cd mouse, Cd levels in the 2y-Cd mouse increased significantly in the renal cortex but were unchanged in the liver. Taken together, the concomitant increment of Cd and MT reduction may cause marked nephrotoxicity in the 2y-Cd mouse. It is conceivable that the resistance to Cd-induced tissue toxicity is due to the more efficient induction of MT, resulting in a higher MT level in the liver than in the kidney. However, in this study, MT expression after Cd administration was lower in liver rather than in kidney, suggesting that factors other than MT may participate in the pathogenesis of Cd-induced hepato-nephrotoxicity (Klassen et al. 1999; Ercal et al. 2001).

Cd toxicity may develop depending on various factors, including the duration of exposure, route of exposure, and dose. In general, Cd given via the oral route is much less toxic than when given parenterally, primarily because Cd is poorly absorbed from the gastrointestinal tract (Liu and Klassen 1996; Brzoska et al. 2003; Patrick et al. 2003). There have been various studies on the relationship between the oral dosage of Cd and onset of tissue toxicity (Mitumori et al. 1998; Liu et al. 2000; Oishi et al. 2000; Wlostowski et al. 2000; Shibutani et al. 2001). The provisional tolerable weekly intake (PTWI) for Cd is estimated at 7 μg/kg body wt., in order that levels of accumulated Cd do not exceed 50 μg/g in the renal cortex (WHO 1993). Based on this calculation, the dose of 100 mg/L was chosen in the present study. Mice received as aqueous solution containing 100 mg Cd/L in drinking fluid during almost their lifespan.

Liver cell necrosis was observed in mice treated with over 200 mg/L of Cd for 8 months but not in mice treated with 40 mg/L of Cd (Mitumori et al. 1998). In the present study, hepatotoxicity was less in mice orally administrated 100 mg/L of Cd for 1.5 year and mild to moderated in mice orally administrated 100 mg/L of Cd for 2 years. As was mentioned above, the 2y-Cd mice reduced MT expression compared to the 1.5y-Cd mice. The precise mechanisms for MT reduction were unclear; however, interaction of Cd with Zn and Cu, a powerful MT inducer, should be consider to participate in Cd-induced tissue damages (Oishi et al. 2000; Pourahmad et al. 2003; Noel et al. 2004; Martelli and Moulis 2004; Valko et al. 2005; Hansen et al. 2006).

Zn is well known as the principal metal with anti-Cd toxicity through MT induction (Klassen et al. 1999; Shimoda et al. 2003; Cai et al. 2005; Martelli et al. 2006). In agreement with previous reports (Oishi et al. 2000; Jurczuk et al. 2004), the net Zn count ratio was increased by oral Cd exposure in both the renal cortex and liver. With regard to the subcellular distribution of Zn in human liver and kidney, Satarug et al. (2001) revealed that Zn was found to exist in at least two pools; one was associated with Cd that bound to MT and the other was associated with non-MT-bound Cu. In the 1.5y-Cd mouse, a large amount of Cd was spatially co-localized with Zn in the liver and renal cortex, thereby free Cd was slightly observed. By contrast, free Cd became abundant in the 2y-Cd-exposed mice, although a relative amount of Cd was combined with Zn. Free Cd was assumed to be responsible for its toxicity (Klassen et al. 1999; Partrick 2003); therefore, Zn may play a protective role in Cd toxicity through MT induction in the 1.5y-Cd mouse.

One of the specific changes that lead to tissue damage and death in chronic Cd exposure is related to oxidative stress. The prooxidative effect of Cd is mediated by Fe (Casalino et al. 1997; Watjen et al. 2004; Martelli and Moulis 2004). Consistent with prior findings (Oishi et al. 2000; Noel et al. 2004; Jurczuk et al. 2004; Swiergosz-Kowalewska et al. 2006), the net Fe count ratio was decreased in the Cd-exposed liver, which may result in mild liver injury. Interaction between Cd and Fe metabolism in the kidney is not fully understood (Oishi et al. 2000; Jurczuk et al. 2004). X-ray spectra from the Cd-exposed mice revealed that Fe was slightly accumulated in the renal cortex, in which a small amount of Cd was co-localized with Fe. These data speculate the participation of Fe in Cd toxicity, although further studies are needed to clarify the role of Fe in Cd-induced hepato-nephrotoxicity.

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